Selective Induction of Immune Responses by Cytokines Expressed in Recombinant Poxvirus Vectors

by

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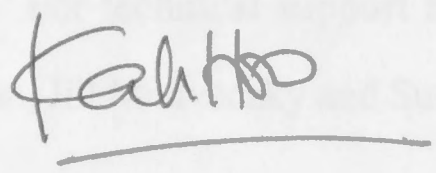
STATEMENT

Unless stated below, the work presented in this dissertation was performed by myself. Recombinant fowlpox viruses were constructed in collaboration with Dr. David Boyle at the Australian Animal Health Laboratory, Geelong, and Mrs. Jill Medveczky at the John Curtin School of Medical Research (JCSMR); FPV insertion plasmids deriving from pAF09 and pAF03 however were made by myself. Evaluations of systemic antigen-specific cytotoxic T lymphocyte and antibody responses involving mice immunised with FPV-HA-IFNγ, in Chapter 3, were performed in collobration with Mrs. Jill Medveczky. In Chapter 4, titration of vaccinia virus was carried out with Mrs. Jill Medveczky and Jianhe Chen, JCSMR.



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For the earth will be filled with the knowledge of the glory of the LORD, as the waters cover the sea.

Habbakuk 2:14

PUBLICATIONS

Work presented in this thesis has been reported in the following publications:

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Ramsay, A.J., K.H. Leong, D.B. Boyle, J.C. Ruby, and I.A. Ramshaw. 1994. Enhancement of specific mucosal IgA responses by interleukins 5 and 6 encoded in recombinant vaccine vectors. *Reproduction, Fertility and Development* **6:**389-392.

Ramshaw, I.A., K.H. Leong, J.C. Ruby, A.J. Ramsay, and D.B. Boyle. 1994. Recombinant viral vaccines expressing cytokine genes. *In* Vaccines 94 (Eds.: E. Norrby, F. Brown, R.M. Chanock and H.S. Ginsberg), pp.29-33. Cold Spring Harbor Laboratory Press, New York.

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ABSTRACT

The generation of antibody and cell-mediated immune responses, which may be mutually exclusive, is critically dependent on the profile of cytokines present during the development of the response. Accordingly, cytokines expressed by recombinant vaccinia virus (VV) have been shown to have profound effects on antiviral immune responses. This thesis describes the selective induction of immune responses by cytokine genes expressed by recombinant VV and fowlpox virus (FPV) and the development of novel vaccine strategies using recombinant FPV and nucleic acid vaccines (NAV) as potentially safe approaches to immunisation.

Avipoxviruses have recently been studied as potential vectors for the delivery of heterologous vaccine antigen. Because these viruses abortively infect mammalian cells, yet still effectively present encoded foreign genes to the host immune system, they offer a safer but effective alternative to other virus vectors. The construction of recombinant FPV expressing influenza virus A/PR/8 hemagglutinin (HA) glycoprotein and the genes for IL-6 or IFN-γ was described. These recombinants secreted cytokines for prolonged periods with minimal cytopathic effect on infected cells *in vitro*. In mice, vector-expressed cytokines dramatically altered immune responses induced against HA. Expression of IL-6 augmented both primary systemic and mucosal antibody responses and primed for enhanced recall responses. When given systemically, IL-6 recombinant virus gave enhanced protection against morbidity upon lethal challenge with influenza virus. In contrast, expression of IFN-γ markedly inhibited antibody responses without affecting the generation of cell-mediated immunity. The safety of FPV was demonstrated in immunodeficient animals and neonates and no side-effects due to cytokine expression were observed.

Intramuscular (i.m.) injections of NAV expressing the HA of influenza virus generated protracted but low levels of specific antibodies in mice. Resultant protection against infection with HA-encoding VV or lethal doses of influenza virus demonstrated the efficacy of NAV vaccination. NAV and FPV, both expressing a common HA antigen,

were combined in a consecutive immunisation strategy in attempts to augment the immune responses elicited by the former. Priming with NAV i.m. and boosting with recombinant FPV at the relevant site generated prolonged and greatly enhanced systemic and mucosal antibody responses. Further enhancement of mucosal antibody responses was obtained when IL-6 was co-expressed in the recombinant FPV. The efficacy of this combined vaccine strategy was further emphasised by its ability to protect mice against both mortality and morbidity upon lethal challenge with influenza virus.

Attenuation of VV has previously been achieved by the expression of cytokines, including IL-2, IFN-γ and TNF. Localised production of these virus-encoded factors during virus replication has also facilitated investigations into their antiviral activities. The attenuation of VV expressing IL-7 in both normal and immunodeficient mice, together with *in vivo* effects of IL-7 expression on antiviral responses were reported. Virus-encoded IL-7 induced elevated LAK and specific CTL responses but did not augment specific antibody levels. The selective enhancement of cell-mediated immune responses by virus-encoded IL-7 was mediated by host IL-2.

In summary, poxvirus vectors encoding cytokines may represent a safe and effective vaccine strategy and can be used to selectively manipulate the immune response. The efficacy of these vectors was further enhanced by combining vaccine strategies using recombinant FPV and NAV. These findings have implications for the development of improved vaccination strategies.

ABBREVIATIONS

Most of the abbreviations used in this thesis are standard; however, attention is drawn to the following:

ADCC	antibody dependent cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
APC	antigen presenting cells
ASC	antibody-secreting cells
^{o}C	degree Celsius
CES	chicken embryo skin
Ci	Curie
CMV	cytomegalovirus
con A	concanavalin A
CPE	cytopathic effects
cpm	counts per minute
CPV	canarypox virus
CTL	cytotoxic T lymphocytes
E:T	effector per target ratio
Ecogpt	xanthine-guanine phosphoribosyl transferase gene of *Escherichia coli*
env	envelope
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
EV	ectromelia virus
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
Fig.	Figure
FITC	fluorescein isothiocyanate
FPV	fowlpox virus
g	gramme
GKO	gene-knockout
GM-CSF	granulocyte-macrophage colony-stimulating factor
gp	glycoprotein
h	hour
HA	hemagglutinin
HAU	hemagglutinating unit
HIV	human immunodeficiency virus
HSV	herpes simplex virus
i.d.	intradermal
i.m.	intramuscular

i.n.	intranasal
i.v.	intravenous
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kb	kilobase
l	litre
*lac*Z	β-galactosidase gene
LAK	lymphokine-activated killer
LCMV	lymphocytic choriomeningitis virus
LD_{50}	lethal dose for 50% of test animals
M	molar
m	metre
MHC	major histocompatibility complex
MHV	murine hepatitis virus
min	minute
MLC	mixed lymphocyte culture
moi	multiplicity of infection
mRNA	message RNA
NAV	nucleic acid vaccine
ND	not determined
NK	natural killer
NP	nucleoprotein
ORF	open reading frame
%	percentage
P.E/L	early/late promoter of fowlpox virus
P7.5	early/late promoter of vaccinia virus
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFU	plaque-forming unit
RE	restriction endonuclease
rpm	rotations per minute
SCID	severe-combined immunodeficient
SD	standard deviation
sec	second
SEM	standard error of the mean
TCR	T cell receptor
TGF	tumour growth factor
Th	T helper

TK	thymidine kinase
TNF	tumour necrosis factor
v/v	volume per volume
VSV	vesicular stomatitis virus
VV	vaccinia virus
W	Watt
w/v	weight per volume
wk	week

CONTENTS

CHAPTER 1

Introduction and Literature Review

1.1 OVERVIEW

The immune system comprises adaptive and innate components. Cytotoxic T lymphocytes (CTL) and antibodies are major elements of the former, whereas innate components include natural killer (NK) cells, macrophages, complement and cytokines (Male and Roitt, 1993). Invasion of foreign materials, often microbes, activates the immune system which in attempts to eliminate the invader. The first line of defence is usually the activation of non-specific innate immunity. Adaptive defences are elicited later and are mediated through two distinct but frequently overlapping modes: generation of CTL and production of antibodies by B lymphocytes. The innate and adaptive systems function in an integrated manner. For example, innate cellular components may interact with specific antibodies in antibody-dependent cell-mediated cytotoxicity (ADCC).

After the initial encounter with infectious agents, the adaptive immune system acquires memory that allows the induction of secondary immune responses faster and at greater amplitude than primary responses. Such enhanced secondary responses prevent the agent from causing disease following subsequent infections. This ability to establish immunologic memory is, therefore, important for protection against virulent infections, as well as for modification of diseases. Thus, the manipulation of the immune system to induce protection has important ramifications for the development of prophylactic and therapeutic vaccines.

1.2 IMMUNITY TO VIRUSES

Antiviral defences are both innate and specific, and their major purpose is to inactivate free virus and to eliminate infected cells that have potential to release infectious virions. Antibodies, which will be discussed further in Sections 1.2.4 and 1.4, are produced by B lymphocytes and neutralise infectivity of virus. Initial resistance to virus infection, however, is mediated by the activation of non-specific NK cells (Bancroft *et al.*, 1981; Borysiewicz *et al.*, 1985; Rager-Zisman *et al.*, 1987) and macrophages (Zisman *et al.*, 1971). Subsequent activation of CTL may lead to the destruction of virus-infected cells and recovery from the virus infection (Gardner *et al.*, 1974; Blanden and Gardner, 1976; Pfizenmaier *et al.*, 1977; Moskophidis *et al.*, 1987; Owen *et al.*, 1988). ADCC is

another mode of killing of virus-infected cells that are bound with antibodies. ADCC has important antiviral roles in resistance to herpes simplex virus (HSV) infections (Kohl and Loo, 1982) and for clearance of a retrovirus, Friend leukemia virus (Collins *et al.*, 1983). In addition, the destruction of virus-infected cells is also mediated by complement proteins that bind to antibodies on infected cells (Hicks *et al.*, 1978; Cooper and Nemerow, 1986). In order for the immune system to respond to virus infections, the immune cells must first be able to 'see' and recognise the pathogen, and in turn, be activated to a variety of antiviral effector functions.

1.2.1 Antigen processing and recognition

Unlike antibody, which recognises intact proteins, T lymphocytes recognise peptides in association with host major histocompatibility complex (MHC) molecules (Zinkernagel and Doherty, 1974). Later, Townsend *et al.* (1985) showed that virus-specific CTL recognises only fragments of the viral antigen and not the whole protein.

There are two classes of MHC molecules which differ in many respects, including structure, composition and the nature of the peptide they bind (Cresswell, 1994). Class I MHC is found on all nucleated cells, whereas only certain types of cells, such as antigen-presenting macrophages, dendritic cells and B lymphocytes, express class II molecules (Harding, 1991). The recent elucidation of structures for class I (Zhang *et al.*, 1992) and class II (Brown *et al.*, 1993) MHC molecules has led to further insights into mechanisms of antigen processing and presentation.

Class I MHC-restricted $CD8^+$ CTL recognise antigens synthesised within target cells (Townsend *et al.*, 1986 and 1989; O'Rourke *et al.*, 1990). These antigens are degraded in the cytosol by proteosomes which consist of multiple subunits of low molecular mass polypeptides encoded by genes in the MHC (Goldberg and Rock, 1992). The peptide fragments are translocated by specific transporter molecules (Monaco, 1992) into the endoplasmic reticulum (ER) where the MHC molecules are derived (Townsend *et al.*, 1989; Neefjes *et al.*, 1993). The peptides then bind to the class I MHC molecules,

and are transported to the Golgi apparatus and ultimately to the target cell surface. Class II MHC-restricted $CD4^+$ helper T (Th) lymphocytes, on the other hand, recognise exogenously ingested antigens (Nelson *et al.*, 1992). A key feature of this pathway is the transient association of class II MHC molecules with another molecule, called the invariant chain, in the ER (reviewed by Cresswell, 1994). The contribution of the invariant chain in antigen processing is not clearly defined. Nevertheless, upon binding of degraded peptides of the internalised protein to the invariant chain-class II MHC molecules in the ER, the invariant chain is cleaved from the complex. The peptide-class II MHC is subsequently presented on the cell surface.

The presentation of endogenous and exogenous antigens by class I and II MHC molecules, respectively, may not be true for all antigens. For example, influenza virus A matrix-specific CTL (Nuchtern *et al.*, 1990) and measles virus-specific CTL (Sekaly *et al.*, 1988) recognise these antigens in association with class II MHC molecules. This peculiar class II MHC-restricted cytotoxicity will be detailed in Section 1.2.3.3.

1.2.2 Lymphocyte activation

T lymphocytes recognise specific peptide-MHC molecules on antigen presenting cells (APC) via a T-cell-surface heterodimer receptor (TCR) and this interaction dictates immunological specificity. Contact between the TCR and the peptide-MHC molecules alone, although essential to initiate activation of T lymphocytes, is insufficient to trigger subsequent effector functions (Mueller *et al.*, 1989). Accessory molecules, including ICAM (Van Seventer *et al.*, 1990; Damle *et al.*, 1992), LFA-3 (Moingeon *et al.*, 1989) and VCAM-1 (Damle and Aruffo, 1991) are required for co-stimulatory interactions between T lymphocytes and APC. The T lymphocyte co-receptors CD4 and CD8 are further important accessory receptors that play distinct but important roles in T lymphocyte activation and effector functions. Generally, $CD4^+$ lymphocytes are of the helper phenotype while $CD8^+$ lymphocytes are cytotoxic (Collins *et al.*, 1993). A 'second signal', provided by interactions between co-stimulatory molecules B7-1, B7-2, or B7-3 on APC with CD28, has recently been shown to be vital for T lymphocyte activation (Galvin *et al.*, 1992; Goldbach-Mansky *et al.*, 1992; Azuma *et al.*, 1993; Freeman *et al.*, 1993;

Harding and Allison, 1993; Van Gool *et al.*, 1993). Without this second signal, T lymphocyte clonal anergy or deletion may occur, producing a state of immunological tolerance. These 'second signals' appear to stabilise message RNA (mRNA) for interleukin 2 (IL-2), interferon-γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in T lymphocytes (Lindsten *et al.*, 1989), thereby prolonging delivery of activation signals (reviewed by Allison, 1994). Other natural ligands for B7 involved in T lymphocyte activation are CTLA-4 (Lenschow *et al.*, 1993) and heat-stable antigen (Liu *et al.*, 1992). CTLA-4 is also involved in B lymphocyte activation (Linsley *et al.*, 1991). Cytokines are also required for the activation of T lymphocyte effector functions, but this will be discussed elsewhere (Section 1.3.5).

B lymphocyte-antigen recognition is mediated by membrane immunoglobulin (Ig) antigen receptors (reviewed by Reth, 1992; Möeller, 1993; Sakaguchi *et al.*, 1993). The receptor mediates the internalisation of antigen which is subsequently degraded and presented in association with class II MHC molecules on the cell surface (Lanzavecchia, 1990; Hodgkin and Basten, 1995). B lymphocytes usually require stimulatory signals or 'help' from activated $CD4^+$ Th lymphocytes for their activation to antibody production (reviewed by Parker, 1993). During the B-T lymphocyte interaction, Th lymphocytes recognise the peptide-class II MHC molecules on B lymphocytes and, through a variety of cytokines produced, induce B lymphocytes to proliferation, differentiation and subsequent antibody secretion. Cytokines secreted by the T lymphocyte have profound effects on the antibody isotype produced (detailed in Section 1.3.7). The B-T lymphocyte interaction, like the T lymphocyte-APC interaction, is also coupled with interaction between co-stimulatory receptors. These include CD40 (Armitage *et al.*, 1992a; Noelle *et al.*, 1992) and the tumour necrosis factor (TNF)-receptor (Aversa *et al.*, 1993; Macchia *et al.*, 1993) on B lymphocytes that interact with CD40 ligand (CD40L) and TNF-α on T lymphocytes. These interactions enable B lymphocytes to respond to cytokines. $CD8^+$ T lymphocytes have also been shown to provide help to B lymphocytes for antibody secretion (Paganelli *et al.*, 1995). The interaction between $CD8^+$ T lymphocytes and B lymphocytes has not been extensively studied. However, these lymphocytes may be

interacting through their TCR recognising the peptide-class I MHC molecules on B lymphocytes. This TCR-MHC interaction is also coupled with interactions between co-stimulatory molecules, such as the CD40 and CD40L interaction (Lane *et al.*, 1992; Cronin *et al.*, 1995).

1.2.3 Antiviral cell-mediated effector mechanisms

A variety of cells are activated during a virus infection. T lymphocytes, in particular CTL, are crucial for recovery from most virus infections (Blanden, 1970). Although CTL are usually of the $CD8^+$ phenotype, $CD4^+$ CTL are important in some virus infections (Weyand *et al.*, 1989). Other non-specific early-acting cells, such as NK, lymphokine-activated killer (LAK) cells and macrophages are also essential for an effective host defence as detailed below.

1.2.3.1 Natural killer and lymphokine-activated killer cells

NK cells were discovered due to their cytolytic activities against tumour cells. They are distinguished from other lymphoid cells by the absence of B and T lymphocyte antigen receptors: murine NK cells are $CD3^-CD16^+$asialo-$GM1^+NK1.1^+$ (Hackett *et al.*, 1986; Lanier *et al.*, 1988; Welsh *et al.*, 1991). The receptor used by NK cells to recognise tumour or virus-infected cells is not defined. The sensitivity of target cells to NK cytotoxicity was shown to be inversely correlated with their expression of class I MHC molecules (Kärre *et al.*, 1986). There is recent evidence to suggest that NK activity is inhibited when target cells encode a specific natural ligand bound in the class I MHC peptide groove (Storkus *et al.*, 1991). Loss of class I MHC molecules, or their ligands, from the target cell surface render these cells sensitive to NK cell killing (Raulet, 1992). Hence, displacement of self peptides with viral peptides in virus-infected cells, or switching off of cellular MHC expression during virus infection, would confer sensitivity of infected cells to NK lysis.

Antiviral NK cells have been demonstrated in a variety of virus infections, including vaccinia virus (VV; Bukowski *et al.*, 1983; Karupiah *et al.*, 1990b) murine cytomegalovirus (CMV; Bancroft *et al.*, 1981), human CMV (Borysiewicz *et al.*, 1985)

and HSV (Rager-Zisman *et al.*, 1987). Adoptive transfer of NK cells to suckling mice lacking NK activity protected the mice against a normally fatal infection with murine CMV (Bukowski *et al.*, 1985). Depletion of NK cells by treatment with anti-asialo-GM1 antibody resulted in an enhanced virus growth after infection with VV (Bukowski *et al.*, 1983; Karupiah *et al.*, 1990b), murine CMV and murine hepatitis virus (MHV; Bukowski *et al.*, 1983). NK cells are activated early in virus infection, probably through the induction of interferons (Gidlund *et al.*, 1978). NK cells mediate their antiviral activities through killing of virus-infected cells directly or through ADCC (Lanier *et al.*, 1988; Ravetch and Kinet, 1991) and by the production of antiviral cytokines such as IFN-γ (Karupiah *et al.*, 1990a).

LAK cells were first recognised by their ability to lyse NK-resistant tumour cells (Grimm *et al.*, 1982). After incubation with IL-2, mononuclear cells acquire this ability, hence this function is defined as 'lymphokine-activated' killer cell activity and is a form of non-MHC-restricted cytotoxicity. Inasmuch as the receptors responsible for NK activity have proved elusive, structures mediating LAK activity also remain poorly characterised. However, several surface adhesion molecules, such as CD2, ICAM-1 and LFA induced by IL-2, may be involved (Robertson and Ritz, 1992). Upon activation, LAK cells have also been shown to secrete IFN-γ and IL-2 (Moretta *et al.*, 1986). LAK activities can also be induced by phytohaemagglutinin (PHA; Moretta *et al.*, 1986), and cytokines such as IL-4 (Tunru *et al.*, 1993) and IL-7 (Alderson *et al.*, 1990; Hickman *et al.*, 1990; Lynch and Miller, 1990; Naume and Espevik, 1991; Stotter *et al.*, 1991; Kos and Müllbacher, 1992). LAK cells were once thought to be a distinct cell lineage (Grimm *et al.*, 1983). However, some LAK cells were shown to express αβ or γδ TCR and may, therefore, be of the T lymphocyte lineage (Moretta *et al.*, 1986).

The antiviral role of LAK cells has not been extensively studied. One study has shown that LAK cells transferred to NK cell-deficient suckling mice can control the growth of murine CMV (Bukowski *et al.*, 1988). However, in the same report, LAK cells showed no antiviral activities against lymphocytic choriomeningitis virus (LCMV). LAK

cells have also been found at sites of infections with VV and MHV, but the antiviral role of LAK is not established in these cases (Natuk *et al.*, 1989).

1.2.3.2 Macrophages

Macrophages have very important early antiviral functions. This early role has been demonstrated by increased susceptibility to infection with yellow fever virus as a result of macrophage depletion just before, but not after, the virus infection (Zisman *et al.*, 1971). Depletion by silica or specific antibodies also exacerbates HSV infection (Zisman *et al.*, 1970). The mechanisms by which macrophages mediate early antiviral effects are unclear. Macrophages are a source of cytokines (Nathan, 1987), such as IFN-γ and TNF, which have potent antiviral activities. Macrophages also mediate antiviral activities through production of virucidal reactive oxygen intermediates (ROI; Chase and Klebanoff, 1992). Another molecule secreted by activated macrophages is nitric oxide (NO; Ding *et al.*, 1988). Antiviral activities of NO have been demonstrated against ectromelia virus (EV) *in vivo* (Karupiah *et al.*, 1993) and HSV *in vitro* (Croen, 1993; Karupiah *et al.*, 1993). The *in vivo* antiviral effects of NO in VV infection were less marked (M. Rolph, JCSMR, personal communication), although *in vitro* antiviral activities of NO on VV have been demonstrated (Harris *et al.*, 1995). NO has been shown to suppress the catalytic activity of enzymes that are dependent on iron and sulphur prosthetic groups, such as reductases, by formation of nitrosyl-iron-sulphur complexes (Lancaster and Hibbs, 1990; Pellat *et al.*, 1990; Nathan, 1992). Inhibition of ribonucleotide reductase, required for DNA synthesis, by NO may result in blockade of virus replication (Kwon *et al.*, 1991; Lepoivre *et al.*, 1991). Macrophages have also been shown to mediate antiviral activities through ADCC, for example in the killing of togavirus-infected cells (Macfarlan *et al.*, 1977).

Together, the non-specific activation of NK, LAK cells and macrophages provides a basic host defense mechanism against virus infections before activation of the more specific T lymphocyte functions.

1.2.3.3 Cytotoxic T lymphocytes

Recovery from most virus infections is critically dependent on T lymphocytes, in particular CTL. Antiviral CTL responses are usually measurable by 5-8 days, by which time virus has spread to the lymph nodes or spleen. Thereafter, viral titres usually start to decrease. This is certainly the case following infection by EV (Gardner *et al.*, 1974; Blanden and Gardner, 1976), LCMV (Moskophidis *et al.*, 1987) or influenza virus (Pfizenmaier *et al.*, 1977; Owen *et al.*, 1988).

CTL activity has usually been related to class I MHC-restricted cytotoxicity (Townsend *et al.*, 1985 and 1986) although class II MHC-restricted CTL have also been demonstrated. The relative importance of the latter is unclear, given that class II MHC molecules are present only on a limited number of cell types (Harding, 1991). Class II MHC-restricted cytotoxicity against LCMV has been demonstrated in β_2-microglobulin-gene-knockout mice (β_2m-GKO) lacking class I MHC (Muller *et al.*, 1992). Class II MHC-restricted CTL activities towards influenza virus (Kaplan *et al.*, 1984), HSV (Yasukawa and Zarling, 1984) and measles virus (Van Binnendijk *et al.*, 1989) in human, and towards influenza virus (Lukacher *et al.*, 1985), vesicular stomatitis virus (VSV; Browning *et al.*, 1990) and MHV (Heemskerk *et al.*, 1995) in mice have also been described. Although $CD4^+$ and $CD8^+$ CTL against influenza virus have similar cytolytic abilities *in vitro* (Maimone *et al.*, 1986), cytotoxicity mediated by LCMV-specific $CD4^+$ CTL occurs at a lower level than $CD8^+$-mediated cytotoxicity *in vivo* (Muller *et al.*, 1992).

CTL mediate their cytolytic activity through perforin in a calcium (Ca)-dependent manner or through interactions with Fas antigen via a Ca-independent mechanism (reviewed by Squier and Cohen, 1994; Liu *et al.*, 1995). Perforin is produced by $CD4^+$ CTL (Yasukawa *et al.*, 1993), $CD8^+$ CTL and NK cells (Shinkai *et al.*, 1988; Liu *et al.*, 1989; Lowrey *et al.*, 1989). This molecule may also act as a conduit for other molecules, such as granzymes, which in turn, will trigger cell death by apoptosis (Shi *et al.*, 1992; Heusel *et al.*, 1994). The role of perforin in cell-mediated cytolysis has been

substantiated by impairment of cytotoxic effector functions in mutant mice deficient in this protein, leading to their failure to clear LCMV (Kagi *et al.*, 1994).

In some cases however, perforin expression cannot be detected by PCR during target cell killing (Helgason *et al.*, 1992) and some CTL hybridomas are completely devoid of perforin (Berke *et al.*, 1993). The absence of perforin expression in these killer cells has raised the question of whether perforin is essential for their cytolytic activity. Recently, it was shown that a transmembrane signalling mechanism involving a target cell surface APO-1 molecule (also called Fas) and its ligand on CTL (Dhein *et al.*, 1992) kills target cells in a Ca-independent manner (Rouvier *et al.*, 1993) through apoptosis (Dhein *et al.*, 1992). Further cell-mediated killing mechanisms have been suggested, for example cell killing through a novel cytotoxin described by Liu *et al.* (1987). Thus, cytotoxic cells may be equipped with multiple cytotoxic mechanisms to kill a wide range of target cells.

1.2.3.4 Helper T lymphocytes

$CD4^+$ T lymphocytes are activated during virus infections and may provide 'help' for CTL and B lymphocytes. Recently, these helper activities have been correlated to types of cytokines secreted by Th lymphocytes (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989b; detailed in Section 1.3.5). Helper activity, nevertheless, does not appear to be limited to $CD4^+$ T lymphocytes. It can be mediated by cells with other CD4-like molecules, such as LAG-3 (Locksley *et al.*, 1993), or by $CD8^+$ T lymphocytes (Paganelli *et al.*, 1995) through interaction of CD40L on the T lymphocyte with CD40 on B lymphocytes (Cronin *et al.*, 1995).

The requirement for $CD4^+$ T lymphocyte help in induction of antiviral CTL responses is controversial. *In vivo* $CD4^+$ lymphocyte depletion has suggested that such help is important for induction of CTL responses to Moloney Sarcoma virus (Weyand *et al.*, 1989), LCMV (Leist *et al.*, 1989b), VV (Zinkernagel *et al.*, 1978; Leist *et al.*, 1989b) and EV (Karupiah *et al.*, 1995). In contrast, other reports using $CD4^+$ lymphocyte depletion during EV and VV (Buller *et al.*, 1987), and LCMV (Ahmed *et al.*, 1988) infections suggested that T help is not to be necessary for the induction of antiviral CTL

in vivo. T help has also been shown not to be needed for CTL generation against influenza virus by *in vitro* depletion of $CD4^+$ lymphocytes (Liu and Müllbacher, 1989). In addition, Bennink and Doherty (1978) have shown that no help was required for generation of anti-VV CTL. The reasons for the discrepancies in these reports are not immediately apparent but may be due to differences in virus virulence or in technical approaches, such as the time of $CD4^+$ lymphocyte depletion. In circumstances that are independent of Th lymphocyte, 'help' may have come in other forms, such as IL-2 produced by $CD8^+$ T lymphocytes (Mizuochi *et al.*, 1989), or help provided by activated B lymphocytes (Liu and Müllbacher, 1989). Although the requirement of Th activity in the induction of anti-LCMV CTL responses remains unclear, help is necessary for LCMV-specific antibody production (Ahmed *et al.*, 1988).

Another antiviral mechanism mediated by $CD4^+$ T lymphocytes is through induction and secretion of virucidal cytokines, such as IFN-γ (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989a). As well as providing help to CTL and B lymphocytes, $CD4^+$ T lymphocytes may also have direct antiviral cytotoxic effects, such as that shown against influenza virus-infected cells (Ada and Jones, 1986). Other examples of $CD4^+$ class II MHC-restricted cytotoxicity have been discussed in the previous section (Section 1.2.3.3). Taken together, it seems that $CD4^+$ T lymphocytes not only mediate antiviral effector functions by providing help for the induction of CTL and protective antibodies, but also by release of cytokines and by direct cell cytotoxicity.

1.2.4 The antiviral roles of antibody and complement

Antibodies have profound effects on virus replication and virus-infected cells, particularly in the prevention of infection. They may reduce infectivity of virus particles by preventing virus aggregation and attachment to specific cell receptors (Campbell and Cords, 1983; Dietzschold *et al.*, 1983). IgG and IgM are predominantly responsible for neutralisation of free virus particles in serum. IgM responses are independent of help from Th lymphocytes; indeed, IgM secretion is induced following virus infections, for example VSV, coxsackie B1 virus, togavirus and encephalomyocarditis virus (EMCV), in T lymphocyte-deficient nude mice (Burns *et al.*, 1975). IgM is produced during primary

but not secondary immune responses, hence production of IgM is correlated with primary infections (Charan and Zinkernagel, 1986). Some IgM responses will switch to IgG with help from Th lymphocytes, probably through the activities of cytokines (Section 1.3.7). While IgM and IgG are important in serum, IgA antibodies are the major subclass contributing to antiviral functions at the mucosae (Mestecky and McGhee, 1987). This will be reviewed in Section 1.4. Other antiviral effects of antibodies include interference of virus replication by cross-linking of antibody with viral determinants expressed on the cell surface, as was shown in measles-virus-infected cells (Joseph and Oldstone, 1974; Fujinami and Oldstone, 1979). The killing of virus-infected cells can also be mediated by antibodies through ADCC, as well as binding of complement to antibody on virus infected cells leading to the triggering the classical pathway of complement-mediated cytotoxicity.

The innate complement system consists of nearly 30 interacting glycoprotein subunits (reviewed by Campbell *et al.*, 1988). The classical pathway of complement is activated when the C1q subunit binds to IgG or IgM and triggers a cascade of interactions among the other subunits. The alternative pathway is independent of antibody and is mediated by direct cleavage of C3 (reviewed by Müller-Eberhard, 1986). There are reports demonstrating the roles of complement in natural resistance, for example in resistance to LCMV (Welsh *et al.*, 1976b) and retroviruses, such as oncornaviruses (Welsh *et al.*, 1975 and 1976a; Cooper *et al.*, 1976). In resistance to the latter, complement binds directly to viral proteins and activates the classical pathway (Cooper *et al.*, 1976; Welsh *et al.*, 1976a). Lysis of LCMV, however, was mediated by antibody (Welsh *et al.*, 1976b). Together, this information suggests that virus neutralisation by antibody and complement, in general, occurs through blanketing virus from its receptors, reduction of infectivity by complement-mediated virus aggregation, direct lysis of virions, opsonisation by phagocytic cells and killing of infected cells by ADCC.

1.3 CYTOKINES

Cytokines are hormone-like molecules and have many common characteristics, including low molecular weight, transient and local production, and potent activity (Poo *et al.*, 1988; Kupfer *et al.*, 1991). Cytokines play major roles in regulating immunological

activity during an immune response: they interact in a network by inducing or suppressing each other, transmodulating cytokine surface receptors and acting on cell functions (Balkwill and Burke, 1989; reviewed by Kelso, 1989). Many different cytokines are involved in host responses to virus infections, directly or indirectly, with both beneficial and inhibitory effects; however only those having particular relevance to the work described in this thesis will be discussed here.

1.3.1 Interferon-γ

IFN-γ is secreted by $CD8^+$ T lymphocytes (Jassoy *et al.*, 1993; Ruby *et al.*, 1993), some $CD4^+$ T lymphocytes (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989a) and NK cells (Cuturi *et al.*, 1989). IFN-γ was first described as having antiviral activity in homologous cells (Isaacs and Lindenmann, 1957) and these activities will be detailed in Section 1.3.4. Subsequently, this factor was shown to mediate a range of immunoregulatory activities, including activation of macrophages and NK cells, and antibody production (Dijkmans and Billiau, 1988). IFN-γ up-regulates both class I and class II cellular MHC expression (Wong *et al.*, 1983) and thus plays an important role in enhancing adaptive immunity. Increased expression of class I MHC enhances recognition of target cells by CTL, while augmented class II MHC expression may elevate presentation of antigen by APC to Th lymphocytes. IFN-γ also induces macrophages (Dijkmans and Billiau, 1988) to antiviral activity through the production of NO, ROI and TNF, as discussed earlier (Section 1.2.3.2). NK cell killing activity is also augmented by IFN-γ (Weigent *et al.*, 1983). IFN-γ has been shown to affect expression of other cytokines and cytokine receptors, for example in enhancing TNF receptor expression (Ruggiero *et al.*, 1986; Tsujimoto *et al.*, 1986), as well as up-regulating the expression of its own gene (Hardy and Sawada, 1989). The pivotal role of IFN-γ in the regulation of Th lymphocyte subsets and antibody production will be discussed in Sections 1.3.5 and 1.3.7.

1.3.2 Interleukin 6

IL-6 is produced by a variety of cells including fibroblasts, monocytes, T lymphocytes, B lymphocytes, mast cells and keratinocytes. It is a pleiotropic factor that

has different activities on different cell populations (Van Snick, 1990). Indeed, the multiple functions of IL-6 are reflected by the names that have been used to described this factor: an antiviral factor, IFN-β_2, B-cell stimulatory factor, hybridoma/plasmacytoma growth factor, hepatocyte-stimulating factor and CTL differentiation factor (Van Snick, 1990). IL-6 is not produced constitutively but is expressed upon virus infection (Sehgal *et al.*, 1988), activation with lipopolysaccharides (Ulich *et al.*, 1991) or activation by other cytokines (Van Snick, 1990). The receptor for IL-6 is expressed constitutively on resting T lymphocytes; however, in B lymphocytes, its expression occurs only upon activation (Taga *et al.*, 1987). IL-6 is a late acting factor for B lymphocytes enhancing their terminal differentiation (Kishimoto and Hirano, 1988). Muraguchi *et al.* (1988) have shown the enhancing effect of IL-6 on Ig production in cultures of peripheral blood mononuclear cells incubated with the cytokine was only apparent after 4 days. The impact of IL-6 in antibody production will further be discussed in Section 1.3.7.

The activity of IL-6 on T lymphocytes appears to occur in synergy with IL-1 (Van Snick, 1990). This synergy is observed in the induction of CTL responses in accessory cell-depleted allogeneic-mixed lymphocyte cultures where IL-6 enhanced IL-2 responsiveness of the $CD8^+$ T lymphocytes (Renauld *et al.*, 1989).

1.3.3 Interleukin 7

IL-7, a stromal cell-derived cytokine, is another factor having pleiotropic effects on lymphoid cells of both B and T lineages, NK cells and monocytes. Besides its detection in bone marrow stromal cells, mRNA for IL-7 has also been found in liver, kidney, spleen, thymus (Namen *et al.*, 1988a; Wiles *et al.*, 1992) and keratinocytes (Heufler *et al.*, 1993). IL-7 was first described as a growth factor for precursor B lymphocytes *in vitro* (Namen *et al.*, 1988a and 1988b). In addition to its effects on B lymphocyte proliferation, IL-7 may play an important role in both pre-T lymphocyte development as well as on mature T lymphocytes (Morrissey *et al.*, 1989; Murray *et al.*, 1989; Welch *et al.*, 1989; Varma *et al.*, 1990; Tushinski *et al.*, 1991). IL-7 also acts as a co-factor in promoting V(D)J rearrangement of TCR β gene in fetal thymocytes (Muegge *et al.*, 1993), and plays important roles in modulating the early stages of both B and T lymphopoiesis (Henney,

1989). The up-regulation of αβ and γδ TCR expression by IL-7 has also been reported (Appasamy, 1992; Muegge *et al.*, 1993; Uehira *et al.*, 1993).

IL-7 enhances the generation of CTL (Alderson *et al.*, 1990; Hickman *et al.*, 1990; Kos and Müllbacher, 1992), LAK (Alderson *et al.*, 1990; Hickman *et al.*, 1990; Lynch and Miller, 1990; Naume and Espevik, 1991; Stotter *et al.*, 1991; Kos and Müllbacher, 1992) and NK (Naume and Espevik, 1991) cell activities *in vitro*. Recombinant IL-7, when given to mice, augments splenic $CD8^+$ T lymphocytes, thereby altering the $CD4^+/CD8^+$ ratio (Faltynek *et al.*, 1992; Komschlies *et al.*, 1994). IL-7 also influences the proliferation of cells of the myeloid lineage (Faltynek *et al.*, 1992) and induces secretion of IL-1α, IL-1β, IL-6 and TNF-α by monocytes (Alderson *et al.*, 1991). Other activities of IL-7 include potent co-stimulation of the adhesion pathway involving CD2 and CD28 (Costello *et al.*, 1993); this factor was later revealed to induce B7/BB1 expression (Yssel *et al.*, 1993). IL-7 has also been linked to the pathogenesis of inflammatory skin diseases and cutaneous T lymphocyte lymphoma (Heufler *et al.*, 1993), dermatitis (Uehira *et al.*, 1993), and chronic lymphocytic leukemia (Frishman *et al.*, 1993).

The interactions of IL-7 with other cytokines have not been clearly elucidated. *In vitro* proliferation of LAK cells was reported to be independent of IL-2 (Naume and Espevik, 1991) or dependent on this factor (Morrissey *et al.*, 1989; Okazaki *et al.*, 1989). Induction of LAK cell activity by IL-7 was reported to be inhibited by IL-4 *in vitro* (Stotter and Lotze, 1991), however, Alderson *et al.* (1990) were not able to confirm this finding. IL-7 also stimulates CTL generation *in vitro*, in some cases via IL-2 (Alderson *et al.*, 1990) and in other cases, in the absence of IL-2 (Kos and Müllbacher, 1992; Kos and Müllbacher, 1993; Carini and Essex, 1994). Although IL-7 and IL-1 synergise in stimulating $\gamma\delta^+$ T lymphocytes *in vitro* (Skeen and Ziegler, 1993), the *in vivo* interactions of IL-7 with IL-1, as well as with other cytokines, require further investigation.

1.3.4 Antiviral cytokines

Cytokines such as IFN-γ and TNF-α exhibit antiviral activities: indeed, it was such activity that led to the discovery of IFN-γ (Isaacs and Lindenmann, 1957; Wheelock, 1965). The role of cytokines in antiviral responses has been more clearly defined with the identification and cloning of cytokine genes, the availability of assays to measure cytokine expression, and the development of mutant mice lacking genes for cytokine and cytokine receptor (reviewed by Biron, 1994; Kaufmann, 1994).

The importance of the antimicrobial activities of IFN-γ was demonstrated in mice lacking IFN-γ functions. Mutant mice deficient for the IFN-γ gene (Cooper *et al.*, 1993; Flynn *et al.*, 1993) or the receptor for IFN-γ (Kamijo *et al.*, 1993a and 1993b) were unable to control *Mycobacterium* infection. This is probably due to a lack of IFN-γ-mediated macrophage activation underlying the production of antimicrobial agents, such as NO, ROI and TNF-α, and the lack of IFN-γ-induced class II MHC expression (Kamijo *et al.*, 1993b). Targeted disruption of the IFN-γ gene also renders normally resistant mice susceptible to *Leishmania* infection (Wang *et al.*, 1994a). Infection of LCMV was exacerbated in animals depleted of IFN-γ by anti-IFN-γ antibody treatment (Leist *et al.*, 1989a). In addition, antibodies to IFN-γ abrogated the control of LCMV infection in mice adoptively transferred with immune spleen cells (Klavinskis *et al.*, 1989). Further insights into the antiviral functions of IFN-γ came from experiments in which recombinant VV were constructed to express this factor (Kohonen-Corish *et al.*, 1990). Immunodeficient athymic and γ-irradiated mice normally succumb to infection with VV but are able to rapidly control a recombinant VV expressing IFN-γ. The authors concluded that virus-encoded IFN-γ had a direct antiviral effect. IFN-γ can increase the virucidal effects of macrophages by up-regulating their production of hypochlorous acid (Chase and Klebanoff, 1992), NO synthase (Karupiah *et al.*, 1993) and also by up-regulation of MHC expression (Wong *et al.*, 1983). IFN-γ may also act indirectly at late stages of the replication cycle of human immunodeficiency virus (HIV), by inhibiting post-translational processing of virus structural proteins within infected cells (Yahi *et al.*, 1992) and virion production (Biswas *et al.*, 1992), or by inhibiting syncytium formation

(Wells *et al.*, 1991). In the light of these findings, Ramsay *et al.* (1993) proposed that antiviral effects of CTL may be mediated through focusing potent antiviral cytokines such as IFN-γ at sites of virus replication, thus controlling virus spread to adjacent cells.

TNF was first shown to possess tumouricidal activity (Carswell *et al.*, 1975) but was later found to exhibit potent antiviral activities against VSV (Mestan *et al.*, 1986; Wong and Goeddel, 1986), adenovirus, HSV and EMCV (Wong and Goeddel, 1986). Dramatic antiviral synergy is seen between TNF and IFN-γ in inhibiting replication of some viruses, such as VSV, EMCV, HSV and adenovirus, and inducing an antiviral state in uninfected cells (Wong and Goeddel, 1986; Feduchi *et al.*, 1989). The direct antiviral activity of TNF was established in immunodeficient animals infected with a recombinant VV expressing this factor (Sambhi *et al.*, 1991). In addition, a ligand for belonging to the TNF receptor superfamily and expressed on activated T lymphocytes, CD40L (Armitage, 1994), has been shown to exhibit potent antiviral activity when expressed by a recombinant VV (J. Ruby, JCSMR, personal communication).

Another cytokine with antiviral activity is tumor growth factor β (TGF-β), which may suppress HIV replication in monocyte-macrophage cell lines *in vitro* (Poli *et al.*, 1991). However, a biological antiviral role for TGF-β remains unclear, as this factor has also been shown to overcome the lymphocytotropic restriction seen in many HIV isolates, hence increasing pathogenecity of some HIV isolates (Lazdins *et al.*, 1992).

1.3.5 Role of cytokines in immune class regulation

Encounter with antigens leads to the stimulation of cell-mediated and antibody responses which may be mutually exclusive (Parish, 1972; Bretscher, 1981). The role of cytokines in the regulation of these responses was clarified when Mosmann and colleagues observed that mouse $CD4^+$ T lymphocyte clones can be divided according to two predominant cytokine secretion patterns (Mosmann *et al.*, 1986; reviewed by Mosmann and Coffman, 1989b). Th1 clones produce IL-2, IFN-γ and lymphotoxin, and promote delayed-type hypersensitivity (DTH) reactions characteristic of cell-mediated immunity (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989a). Th2 clones,

conversely, produce IL-4, IL-5 and IL-10, and direct antibody responses (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989a; Sher and Coffman, 1992). There is another population of Th lymphocytes that produces both Th1 and Th2 cytokines, and they are referred to as the Th0 phenotype (Firestein *et al.*, 1989; Street *et al.*, 1990). Th1 and Th2 subsets were also found to cross-regulate: IFN-γ secreted by Th1 lymphocytes inhibits Th2 lymphocyte proliferation (Fernandaz-Botran *et al.*, 1988; Gajewski and Fitch, 1988), whereas IL-4 and IL-10 produced by Th2 lymphocytes down-regulate Th1 responses (Powrie and Coffman, 1993a and 1993b).

The inhibition of Th1 cytokine synthesis by IL-10 (Fiorentino *et al.*, 1989) is an indirect process occurring by suppression of APC/accessory cell function for Th1 clones (de Waal Malefyt *et al.*, 1991; Fiorentino *et al.*, 1991a and 1991b; Ding and Shevach, 1992) through the down-regulation of expression of IL-12 (D'Andrea *et al.*, 1993) and B7 (Ding *et al.*, 1993). In contrast, IL-4 directly inhibits *in vivo* effector functions of Th1 populations, such as production of IFN-γ and DTH (Powrie and Coffman, 1993a and 1993b; Powrie *et al.*, 1994), and the development of Th1 lymphocytes from naive populations (Hsieh *et al.*, 1992; Seder *et al.*, 1992). The development of Th2 lymphocytes *in vitro* is promoted by IL-4 during initial stimulation of resting T lymphocytes with either concanavalin A (con A; Swain *et al.*, 1990) or cross-linked anti-CD3 antibodies (Le Gros *et al.*, 1990). The role of IL-4 in Th2 response development *in vitro* was also established using immune cells from specific TCR-transgenic mice (Hsieh *et al.*, 1992; Seder *et al.*, 1992). The development of *in vivo* Th2-type responses to microbial antigens, such as *Leishmania major* (Sadick *et al.*, 1990; Chatelain *et al.*, 1992) and *Candida albicans* (Romani *et al.*, 1992) has also been shown to be critically dependent on the presence of IL-4. Further evidence that IL-4 is central to the development of Th2 responses *in vivo* came from the demonstration of reduced levels of Th2 cytokines following infection of mice lacking the IL-4 gene with the nematode *Nippostrongylus brasiliensis* (Kopf *et al.*, 1993). More recently, the *in vivo* inhibitory effects of IL-4 on the generation of CTL was demonstrated by Sharma *et al.* (1995): a recombinant VV encoding IL-4 inhibited generation of CTL, down-regulated expression

of mRNA for IFN-γ, IL-2 and IL-12 in splenocytes. However, the effects of IL-4 in promoting Th2 cytokine expression were less convincing in mice expressing the IL-4 as a transgene; these mice have elevated expression of IFN-γ (Platzer *et al.*, 1992).

In contrast to the well-characterised development of the Th2 phenotype, the roles of factors involved in Th1 subset development remain controversial. One of the dominant factors determining Th1 lymphocyte development, leading to cell-mediated immune responsiveness, is IL-12 (Hsieh *et al.*, 1993b; Manetti *et al.*, 1993 and 1994; Seder *et al.*, 1993; Sieling *et al.*, 1994). This factor is produced by monocytes (D'Andrea *et al.*, 1992) and B lymphocytes (Stern *et al.*, 1990), and has been shown to suppress the production of IL-4 (Manetti *et al.*, 1993). Moreover, IL-12 has been found to promote the development of Th1 responses, as shown by the promotion of CTL activity by IL-12 both *in vivo* (Gately *et al.*, 1994) and *in vitro* (Chouaib *et al.*, 1994), and by the induction of T lymphocytes to IFN-γ expression (Seder *et al.*, 1993; Sieling *et al.*, 1994). IL-12 has been shown to act directly on $CD4^+$ T lymphocytes to stimulate IFN-γ production *in vitro* (Seder *et al.*, 1993). IL-12 has even been shown to induce Th2 subsets to transient IFN-γ production *in vitro* (Manetti *et al.*, 1994). When given *in vivo*, IL-12 promotes antimicrobial Th1 activities against *L. major* (Heinzel *et al.*, 1993; Sypek *et al.*, 1993) and toxoplasma (Gazzinelli *et al.*, 1993) through elevation of IFN-γ production by T lymphocytes or NK cells. The up-regulation of IFN-γ production by IL-12 occurs through synergy with IL-2 and results in stabilisation of mRNA transcripts for IFN-γ (Chan *et al.*, 1992).

The induction of IFN-γ by IL-12 suggests that the activity of IL-12 may be mediated through this factor. Indeed, this has been supported by *in vitro* studies. Antibodies against IFN-γ, added to cultures of cells from specific TCR transgenic mice, inhibit the IL-12-induced production of IFN-γ (Macatonia *et al.*, 1993). In contrast, several studies have demonstrated IL-12 activity occurring independently of IFN-γ. Studies using IFN-γ-GKO mice have revealed an IFN-γ independent, IL-12-mediated reduction of IL-4 production during *Leishmania* infection (Wang *et al.*, 1994b). In addition, co-administration of antibodies to IFN-γ and recombinant IL-12 did not abrogate

activities of IL-12 during immune responses to hemocyanin in normal mice (McKnight *et al.*, 1994).

While IL-12 apparently may act both via IFN-γ and independently of this factor, the role of IFN-γ itself in Th1 development is still unclear. The presence of IFN-γ during priming of T lymphocytes from mice transgenic for the ovalbumin-specific TCR *in vitro* did not enhance subsequent IFN-γ production (Seder *et al.*, 1992; Macatonia *et al.*, 1993; Seder *et al.*, 1993), while mice with targeted disruption of the IFN-γ gene have normal levels of CTL responses (Graham *et al.*, 1993). Mice lacking the receptor for IFN-γ also have normal CTL and Th lymphocyte responses (Huang *et al.*, 1993). In contrast, recombinant IFN-γ, when given *in vivo*, favours the production of IFN-γ and reduced pathogenecity of *Leishmania* in susceptible mice (Scott, 1991). Together, the requirement for IFN-γ in promoting Th1 immune responses has yet to be convincingly demonstrated. IFN-γ has, however, been shown to inhibit Th2 lymphocyte responses (Fernandaz-Botran *et al.*, 1988; Gajewski and Fitch, 1988).

Little is known about the role of IL-2 in the development of different Th subsets (Swain, 1991). However, it is clear that this cytokine may act as a growth factor for lymphocytes (Seder and Paul, 1994). Another factor, TGF-β, may play a role in the development of Th1 responses *in vitro*, as shown by its down-regulation of Th2-type cytokine (IL-4 and IL-5) production (Swain *et al.*, 1991; Hsieh *et al.*, 1993a) and promotion of Th1 cytokine (IL-2 and IFN-γ) secretion (Swain *et al.*, 1991). However, TGF-β was also found to inhibit Th1 effector functions during immune responses to *Leishmania* (Barral-Netto *et al.*, 1992).

The nature and quantity of antigen has also influence the development of cell-mediated or antibody responses (Parish, 1972; Bretscher, 1981). Moreover, the genetic background of experimental animal may contribute profoundly to the types of immune responses mounted following infection (Bretscher *et al.*, 1992). Nevertheless, the vital roles played by cytokines in immune class regulation have been amply shown. Given that infections by viruses or parasites are generally controlled by either cell-mediated or

antibody responses, the pattern of host cytokine production may determine the outcome of the infection.

1.3.6 Chronic or acute disease: a matter of cytokine dominance

Pathogens are most effectively controlled by the induction of either a dominant cell-mediated (a Th1 response) or an antibody (a Th2 response) response (reviewed by Scott and Kaufmann, 1991; Sher and Coffman, 1992; Sher *et al.*, 1992; Urban *et al.*, 1992). Hence, the generation of appropriate Th lymphocyte responses is crucial in the resolution of many infections. Dominance of inappropriate Th responses, in some chronic diseases, has resulted in the inability to eradicate invading organisms, thereby exacerbating the disease. For example, a strong DTH (Th1) response in mice during infection by *L. major* results in eradication of the organism. In contrast, certain mouse strains (for example BALB/c) mount a mostly antibody (Th2) response during *L. major* infection and succumb to disseminated disease (Scott *et al.*, 1988; Heinzel *et al.*, 1989). Such dichotomy of immune responses is also seen in human leishmaniasis (Carvalho *et al.*, 1985; Sacks *et al.*, 1987). Another example is human tuberculoid and lepromatous leprosy that are, respectively, accompanied by a strong Th1 response promoting DTH or a weak cell-mediated immunity. Strong DTH eventually clears the bacilli, whereas weak cell-mediated immune responses result in a more severe and persistent disease (Salgame *et al.*, 1991; Yamamura, 1992). IL-12 has now been implicated in the induction of Th1 lymphocytes in lesions of tuberculoid leprosy but not in lesions of lepromatous leprosy (Sieling *et al.*, 1994). In other disease states, such as during helminth infections (Sher and Coffman, 1992; Urban *et al.*, 1992), toxoplasma (Sher and Coffman, 1992; Sher *et al.*, 1992) and listeriosis (Magee and Wing, 1988; Tripp *et al.*, 1993), similar correlations also exist between Th lymphocyte phenotype dominance and disease susceptibility.

It has recently been suggested that a Th2 bias may occur during progression to the Acquired Immunodeficiency Syndrome (AIDS) following infection with HIV (Maggi *et al.*, 1987; Sher *et al.*, 1992; Clerici and Shearer, 1993; Clerici *et al.*, 1993a). HIV causes a slow progressing infection that, initially, is essentially symptomless.

Subsequently, the course of HIV infection leads to a loss of $CD4^+$ T lymphocytes, suppressed CTL levels and susceptibility to other pathogens (Rosenberg and Fauci, 1989). Clerici *et al.* (1993a and 1994b) have noted that peripheral blood cells from HIV-infected individuals secrete reduced levels of IL-2 (a Th1 cytokine), and high levels of IL-4 and IL-10 (Th2 factors) after stimulation with recall antigens, allogeneic cells or PHA. These observations led Clerici and Shearer (1993) to postulate that the progression to AIDS is due to Th2 responses induced in HIV-seropositive individuals. This hypothesis was supported by findings of non-lytic $CD8^+$ lymphocytes secreting Th2 cytokines that provide help to antibody responses (Maggi *et al.*, 1994a) and suppressed IL-12 expression (Chehimi *et al.*, 1994) in HIV-seropositive individuals, and high levels of cell-mediated immunity in seronegative HIV-infected individuals (Clerici *et al.*, 1992).

However, studies of secondary lymphoid tissues in HIV-infected individuals have not confirmed the Th2-bias in HIV infection during progression to AIDS (reviewed recently by Romagnani *et al.*, 1994). There was no demonstrable overall shift towards the Th2 responsiveness shown in the abovementioned *in vivo* studies: expression of IL-4 was not detected (Emile *et al.*, 1994; Graziosi *et al.*, 1994) while IL-2 and IFN-γ expression was found (Emile *et al.*, 1994). Instead, Maggi *et al.* (1994b) have demonstrated a predominance of cells of the Th0 phenotype. The different lymphoid cells used in the abovementioned studies may account for the discrepancies observed. Later, Clerici and Shearer (1994) proposed a new classification for grouping according to cytokine functions, i.e. type-1 or type-2 cytokines, instead of the former dichotomy of Th1-Th2 based on the cell-type producing a particular factor.

An uncontrolled or chronic Th lymphocyte response can also lead to immunopathology, for example in vernal conjunctivitis (Maggi *et al.*, 1991), exposure to allergens (Wierrenga *et al.*, 1990; Parronchi *et al.*, 1991; Yssel *et al.*, 1992) and in infections with respiratory syncytial virus (Alwan *et al.*, 1994) or *Borrelia* (Yssel *et al.*, 1991). Therefore, the ability to manipulate Th lymphocyte phenotypes present following exposure to antigen might not only be advantageous for the development of improved prophylactic strategies, but also raises the possibility of inducing an appropriate response

for a specific pathogen with minimum pathology. The selective manipulation of immune responses with cytokines may have important implications in the design of vaccines for prophylaxis and therapy.

1.3.7 Role of cytokines in antibody production

A number of cytokines secreted by T lymphocytes, during interactions between T and B lymphocytes, may directly affect the development and maturation of B lymphocytes. IL-4 acts early in the activation process (reviewed by Coffman *et al.*, 1988; Snapper *et al.*, 1988; Mosmann and Coffman, 1989a) while the action of IL-5 is apparently restricted to activated B lymphocytes (Beagley *et al.*, 1988; Harriman *et al.*, 1988), suggesting that IL-4 and IL-5 may act in sequence. As well as its activity on T lymphocytes, IL-2 induces proliferation of B lymphocytes (Waldmann *et al.*, 1984) and synergises with IL-5 in enhancing antibody responses *in vitro* (Takatsu *et al.*, 1988). IL-6 is a B lymphocyte differentiation factor (Vink *et al.*, 1988) that acts late in the differentiation process (Kishimoto and Hirano, 1988; Muraguchi *et al.*, 1988). Given this information, it is thought that these cytokines have the following temporal order of activity on B lymphocytes: IL-4 → IL-5 → IL-2 → IL-6 (Sanders and Vitetta, 1990).

Cytokines also contribute to the molecular mechanism of isotype switching although the signals that control the switch are not fully understood. IFN-γ favours the production of IgG2a in mice as illustrated by Snapper and Paul (1987), and by Finkelman *et al.* (1990). IL-4 is important for the production of IgG1 and IgE (Snapper *et al.*, 1988; Finkelman *et al.*, 1991; Goodman *et al.*, 1993) and may be crucial for the latter (Finkelman *et al.*, 1991). With the development of IL-4-GKO mutant mice, IL-4 was shown to be necessary, but not essential, for the production of IgG1. Low levels of IgG1 were found in these naive mutant mice (Kuhn *et al.*, 1991; Kopf *et al.*, 1993) and in those given (4-hydroxy-3-nitrophenyl)acetyl-chicken γ-globulin (Kuhn *et al.*, 1991) or *N. brasiliensis* (Kopf *et al.*, 1993). Very recently, the effect of IL-4 in development of mucosal antibody responses has also been studied (Vajdy *et al.*, 1995; Ramsay *et al.*, unpublished). Mice lacking the IL-4 gene have impaired mucosal IgA antibody responses with the defect due to a poor development of Th2 lymphocytes at the mucosae. Cytokines

such as IL-5 (Beagley *et al.*, 1988; Harriman *et al.*, 1988; Ramsay and Kohonen-Corish, 1993) and IL-6 (Beagley *et al.*, 1989; Ramsay *et al.*, 1994a and 1994b), which are secreted by Th2 lymphocytes and other non-T cells, at the mucosae (Taguchi *et al.*, 1990; Mega *et al.*, 1992; Xu-Amano *et al.*, 1992) are particularly important for differentiation of committed IgA B lymphocytes into IgA-secreting cells. Mutant mice lacking IL-6 mount negligible antibody responses to virus infection at the mucosae, however, infection with a recombinant VV expressing IL-6 restored mucosal antibody responses in these animals (Ramsay *et al.*, 1994a). TGF-β, together with IL-4 and IL-5, has also been shown to promote IgA class switching of activated B cells *in vitro* and this activity can be inhibited by IFN-γ (McIntyre *et al.*, 1995). However, the physiological significance of TGF-β-mediated production of IgA remains to be demonstrated, given that IgA secretion under these conditions is only associated with a small percentage of cells with surface IgA (Lebman *et al.*, 1990; Ehrhardt *et al.*, 1992). Together, these data suggest that the manipulation of antibody responses by cytokines may also have important ramifications for the development of vaccine strategies for immunisation of mucosal surfaces.

1.4 THE MUCOSAL IMMUNE SYSTEM

Antibodies in the systemic circulation neutralise infectivity and promote clearance of viruses that have a viremic mode. Indeed, Ig prophylaxis has been shown to prevent or decrease infection of target organs by measles, hepatitis, rubella and varicella viruses (Krugman, 1963). However, the great majority of pathogenic organisms infect the large and exposed mucosal surface area. An immune system which is at least partially autonomous from the systemic immune system has developed to protect these sites. The mucosal immune system is anatomically and functionally distinct, and is divided into inductive and effector tissues (Kiyono *et al.*, 1992; McGhee *et al.*, 1992). Recent increased understanding of the mucosa-associated lymphoid tissues (MALT), reviewed recently by Staats *et al.* (1994), has provided a rationale for improved immunoprophylaxis at the mucosae.

1.4.1 Inductive tissues

The major mucosal inductive sites are the gut-associated lymphoid (GALT) and nasal-associated lymphoid tissues (NALT) that are strategically located at the gastrointestinal (GI) tract and nasopharyngeal area (tonsils). Other MALT, including that of the reproductive tract, will not be reviewed here. Antigenic stimulation of inductive tissues of the MALT, particularly the Peyer's patches (PP) of the gut, gives rise to both mucosal cell-mediated and antibody responses (reviewed by Mestecky and McGhee, 1987; McGhee *et al.*, 1992; McGhee and Kiyono, 1993). Nearly 75% of T lymphocytes in these tissues express $\alpha\beta$ TCR, with a small population of $\gamma\delta^+$ T lymphocytes which remain poorly characterised. Among the $\alpha\beta^+$ T lymphocytes, more than half (50-60%) are $CD4^+$ Th lymphocytes and the rest are $CD8^+$ (Hunninghake and Crystal, 1981). PP also contain APC (phagocytic dendritic cells and macrophages) and B lymphocyte zones (follicles) containing the majority of B lymphocytes with surface IgA. Follicle-associated epithelium or microfold (M) cells allow transport of intact antigen from the apical membrane exposed to the lumen of the GI tract to the basolateral surface and underlying lymphoid cells.

The NALT and bronchus-associated lymphoid tissues (BALT) are mucosal inductive sites of the upper (URT) and lower respiratory tracts (LRT), respectively (Kuper *et al.*, 1992). NALT appears to be the principal inductive site in these tissues, however both are structurally organised lymphoid tissues containing B and T lymphocytes, APC (phagocytic dendritic cells and macrophages), and M cell-like epithelial cells to facilitate antigen processing and induction of immune cells that relocate to distant effector tissues (Fig. 1.1).

Antigen-specific T and B lymphocytes, upon activation, leave inductive sites via efferent lymphatics and are transported to the systemic circulation through the thoracic duct. These lymphocytes ultimately arrive at mucosal effector sites consisting of the lamina propia (LP) of respiratory, GI and reproductive tracts, as well as glandular tissues, such as the salivary and mammary glands. This circular pathway for dissemination of T

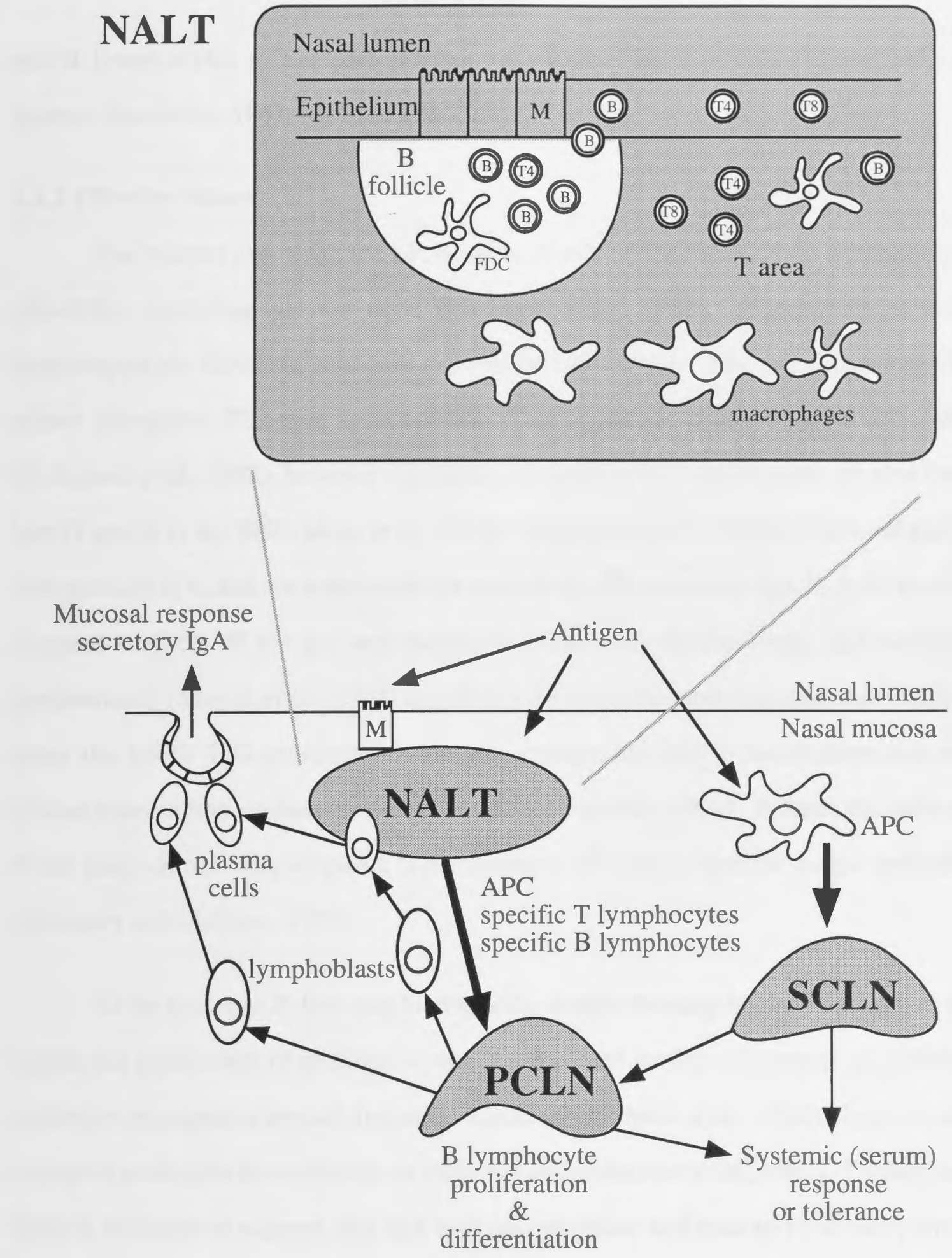


FIGURE 1.1 ***Schematic representation of compartments and cellular composition of NALT***

The pathways eliciting mucosal and systemic responses via NALT are illustrated (Adapted from Kuper *et al.*, 1994)

B, B lymphocytes; T4, $CD4^+$ T lymphocytes; T8, $CD8^+$ T lymphocytes; M, microfold epithelial cells; APC, antigen-presenting cells (macrophages & dendritic cells); NALT, nasal-associated lymphoid tissues; PCLN, posterior cervical lymph node; SCLN, superior cervical lymph node

and B lymphocytes to mucosal effector sites forms the 'common mucosal immune system' (Mestecky, 1987; McGhee *et al.*, 1989).

1.4.2 Effector tissues

The effector site of GI, the LP, consists mainly of T (40-60%) and B lymphocytes (20-40%), including plasma cells (McGhee *et al.*, 1992). About 60% of the T lymphocytes are $CD4^+$ and one-third express the CD8 marker. The major activated CD4 subset comprises Th2-type lymphocytes (Taguchi *et al.*, 1990; Mega *et al.*, 1992; Xu-Amano *et al.*, 1992), however significant numbers of Th1 lymphocytes are also found here (Taguchi *et al.*, 1990; Mega *et al.*, 1992). Approximately 70-90% of the local plasma cells produce IgA, and are responsible for antigen-specific secretory IgA (S-IgA) found at mucosal surfaces of the gut and intestinal secretions. In the lungs, IgA secretion predominates (Merrill *et al.*, 1982) and is thought to be the most important host defense along the URT. IgG production is far less evident, but IgG is found more and may assume roles in host defense in smaller bronchi (Reynolds, 1991). Indeed, the hallmark of the mucosal antibody response is the secretion of antigen-specific S-IgA antibodies (Mestecky and McGhee, 1987).

At the mucosae, S-IgA may bind specific antigen forming immune complexes and inhibit the interaction of pathogens with the mucosal surface (Crowe *et al.*, 1994), a protective mechanism termed 'immune exclusion' (Michetti *et al.*, 1992). IgA can also neutralise pathogens intracellularly in epithelial cells (Mazanec *et al.*, 1992). Furthermore, there is evidence to suggest that IgA may be cytophilic and bind to Fc-α receptors of alveolar macrophages (Sibille *et al.*, 1986). IgA has also been shown to bind complement during the complement-mediated killing of *Neisseria* (Jarvis and Griffiss, 1989). However, the cytophilic activities of IgA remain relatively poorly studied.

Cytotoxicity by CTL (Kagnoff and Campbell, 1974; Guy-Grand *et al.*, 1978; Davies and Parrot, 1981; Issekutz, 1984; Klein *et al.*, 1985; Klein and Kagnoff, 1987), ADCC (MacDermott *et al.*, 1980) and NK cells (Tagliabue *et al.*, 1981) has been described at mucosal effector sites. Antigen-specific CTL activities have been

demonstrated at the mucosae following local administration of allogeneic tumour cells (Kagnoff and Campbell, 1974; Klein *et al.*, 1985; Klein and Kagnoff, 1987) and are mediated by $CD8^+$ T lymphocytes (Ernst *et al.*, 1985; Klein *et al.*, 1985). Infection with rotavirus (Offit and Dudzik, 1989) or VV (Issekutz, 1984) at the mucosae generates local and also systemic CTL in the spleens or adjacent lymph nodes.

1.4.3 Immunity to influenza virus

Immunity to influenza virus infection has been reviewed at length (Potter and Oxford, 1979; Askonas *et al.*, 1982; Couch and Kasel, 1983; Murphy and Clements, 1989). Antibodies mediate immunity to strain- or subtype-specific influenza virus, whereas cross-reactive immunity is cell-mediated (Askonas *et al.*, 1982; Ada *et al.*, 1983; Ada and Jones, 1986). CTL recognise the nucleoprotein (NP) of the virus as a cross-reactive antigen (Yewdell *et al.*, 1985; McMichael *et al.*, 1986; Gotch *et al.*, 1987) and thus clear infection with either heterologous (Taylor and Askonas, 1986; Montgomery *et al.*, 1993) or homologous (Taylor and Askonas, 1986) virus.

The major protective antibody against influenza virus infection is S-IgA directed against the hemagglutinin (HA) glycoprotein of the virus (Renegar and Small, 1991). IgA is most important upon infection of the URT but does not always neutralise the virus (Yanagihara and McIntosh, 1980). When infection occurs throughout the respiratory tract, local IgA and serum-derived IgG both provide protection (Couch *et al.*, 1979; Ramphal *et al.*, 1979; McDermott *et al.*, 1982; Prince *et al.*, 1985; Ada and Jones, 1986). Indeed, passive transfer of IgG in the peritoneum affords protection against virus challenge in the lungs (Prince *et al.*, 1985). Despite these findings, a greater understanding of the immune response to influenza virus and other respiratory pathogens is crucial for the development of more effective immunoprophylaxis.

1.5 IMMUNOLOGICAL MEMORY

Memory lymphocytes allow the immune system to mount a much stronger response, that is both faster and more effective than the primary response, on second encounter with antigen (Male and Roitt, 1993). There are two schools of thought

regarding the longevity of memory lymphocytes: one, that memory lymphocytes are long-lived, and the other, that memory lymphocytes are short-lived in the absence of antigen. In support of the first model, Müllbacher (1994) using adoptive transfer, showed that cytotoxic T memory responses against influenza virus can be maintained for 25 weeks in the absence of the virus. $CD8^+$ ($CD44^{hi}$) CTL memory against LCMV has also been maintained in the absence of the antigen (Lau *et al.*, 1994).

Others have demonstrated that lymphocytes of memory phenotype are short-lived and have a more rapid turnover than naive lymphocytes (Mackay *et al.*, 1990; Michie *et al.*, 1992). These short-lived lymphocytes may have been maintained by reversion to long-lived naive lymphocytes (Sprent, 1993); this 'naive memory' population may be ready to respond to recall antigen. Indeed, lymphocytes of memory phenotype have been shown to acquire cell surface markers that are similar to those found on newly activated lymphocytes (Budd *et al.*, 1987; Akbar *et al.*, 1988; Cerottini and MacDonald, 1989; Powrie and Mason, 1989; Bell and Sparshott, 1990). However, memory-type responses have not been detected amongst these 'naive memory' lymphocytes (Merkenschlager *et al.*, 1988). This may be due to an inability to activate these populations *in vitro*.

The long-term maintenance of immunologic memory mediated by short-lived memory lymphocytes with rapid turnover would suggest a continuous activation of memory lymphocytes with antigens. Recent studies of Gray and Skarvall (1988), and Gray and Matzinger (1991) using adoptive-transfer of memory lymphocytes have indeed demonstrated a requirement for antigen persistence in the maintenance of long-term memory B and T lymphocytes primed with hemocyanin. Maintenance of long-term CTL responses to LCMV has also been shown to require antigen persistence (Oehen *et al.*, 1992). The slow release of antigen-antibody complexes retained in follicular dendritic cells (FDC) in B lymphocyte areas may account for the maintenance of memory lymphocytes by continuously restimulating and expanding memory B lymphocyte clones (Tew *et al.*, 1980; Szakal *et al.*, 1989; Gray, 1992).

Other molecules having a significant influence on cell immortality, such as bcl-2 (Nuñez *et al.*, 1991), may contribute to the long-term survival of memory lymphocytes. Bcl-2 blocks cell death by apoptosis following a variety of stimuli (Strasser *et al.*, 1991), and may be important in saving progenitor and long-lived cells in a variety of cell lineages.

At the mucosae, antigen-specific IgA responses are short-lived and reimmunisation may not always induce higher levels of IgA faster during recall antibody responses (McGhee and Kiyono, 1993). There is some evidence to show that long-lasting immune responses can be generated at the mucosae, for example with cholera toxin (Lycke and Holmgren, 1986; Lycke and Holmgren, 1989), however it is possible that these responses may have been maintained by memory lymphocytes in the blood circulation (Lycke *et al.*, 1987). A continual restimulation of MALT may be required for maintenance of mucosal immune responses. Dendritic cells including FDC in the PP may serve an antigen-retaining role in mucosal inductive sites; however, more studies will be required to determine roles for antigen persistence and IgA immune complexes in these tissues. If antigen persistence is predominantly responsible for the maintenance of memory lymphocytes, then mechanisms underlying their persistence are key parameters in determining the efficiency and duration of protection afforded by vaccines.

1.6 THE PRACTICE OF IMMUNISATION

Vaccination is a deliberate attempt to protect humans against infectious diseases. Although vaccination has a long history, it is only in this century that such practices have become routine. With the exception of safe water, no other modality — not even antibiotics — has had such a major effect on reducing mortality than vaccination. Since Edward Jenner introduced vaccination against smallpox, similar practices have resulted in the control of nine major diseases, at least in certain parts of the world: smallpox, diphtheria, tetanus, yellow fever, pertussis, poliomyelitis, measles, mumps and rubella (Plotkin and Plotkin, 1994). Indeed, as the result of active immunisation against smallpox, the dream of eradicating a virulent pathogen has been fulfilled (World Health Organization, 1980).

1.6.1 A brief history of vaccination

The practice of immunisation stems from observations, in early civilisations, that individuals who survived a disease seldom suffered a second similar clinical infection. In the Roman Empire, the livers of rabies-infected dogs were used for protection against rabies (Wigzell, 1993). Protection against smallpox by transmission of smallpox scabs, called 'variolation', was practised in China in the sixth century A.D. (Wong and Wu, 1932; Hume, 1940; Huard and Wong, 1968). Such efforts were also recorded in the Brahmin caste of Indian Hindus in the 16th century (Major, 1954).

However, vaccination procedures employed today have their roots in the mid-18th century. It was observed then, in several European countries, that milkmaids acquiring infection from cows were protected from smallpox. Edward Jenner, in the late 18th century, proved that previous inoculations with harmless cowpox, a practice now known as 'vaccination', afforded protection against the virulent smallpox (Plotkin and Plotkin, 1994). Unfortunately, it was not until 200 years after Jenner's demonstration that this procedure was to be used for widespread protection of humans from diseases. An intensified global programme of smallpox vaccination was carried out in 1967 and in less than 15 years following the beginning of active immunisation, smallpox virus was eradicated. Truly, such success represents an illustration of the efficacy of vaccination (World Health Organization, 1980).

Louis Pasteur, in the latter half of the 1870s, introduced the concept of 'attenuation' to modify the virulence of a pathogen while retaining its immunogenicity (Plotkin and Plotkin, 1994), thus enabling the use of attenuated strains to induce protection against the virulent pathogen. Attenuation of infectious agents can be achieved in different ways, including serial passages in an unrelated host. For example, virulent rabies was passaged in rabbits, while poliovirus (Sabin and Bougler, 1973) and hepatitis A virus (Provost *et al.*, 1983) in primates species.

Since the contributions of Jenner and Pasteur, numerous vaccines have been developed and used successfully despite initial objections or pessimism. Generally, the

vaccines used were either live, attenuated or inactivated preparations and are known as 'conventional' vaccines. Testimonies to the efficacy of such conventional vaccines are the eradication of smallpox and the great reduction in morbidity from infection by bacterial and viruses, such as tuberculoid bacilli, measles, mumps, polio and rubella.

1.6.2 New technologies for making vaccines

Although conventional vaccines have proved to be most successful for prophylactic immunisation, their use has certain inherent limitations. These include the limited stability of some vaccines under field conditions and the cost of preparation of large quantities of attenuated vaccine strains. The most notorious side-effect is the occasional reversion of attenuated strains to virulent phenotypes giving rise to vaccine-induced or vaccine-associated complications. For example, immunisation with poliovirus (Norrby, 1987) or VV (Lane *et al.*, 1969; Lane *et al.*, 1970; Behbehami, 1983) has been noted to cause complications, particularly in immunocompromised individuals (see also Section 1.7.4). Atypical infections have been related to vaccination with inactivated preparations, ~~such~~ as atypical measles may arise following vaccination with formalin-treated measles virus (Norrby, 1987).

Conventional vaccines were made available without a comprehensive knowledge of crucial properties of the organism or of their effects on the immune system. With the advent of recombinant DNA technology and increased understanding of immunology and protein chemistry, new candidate vaccines with the potential to overcome many of the limitations of conventional vaccines have been developed. Such novel strategies include synthetic peptides (Arnon, 1987), subunit vaccines (Zanetti *et al.*, 1987), anti-idiotype immunogens (Dalgeish and Kennedy, 1988), immunostimulating complexes (Morein *et al.*, 1987; Lövgren *et al.*, 1990) and vaccines based on replicating recombinant bacterial and viral vectors (Cavanagh, 1985; Flexner *et al.*, 1988). Attempts have even been made to express vaccine antigens in transgenic plants with the aim of inducing systemic and mucosal immunity using 'edible' vaccines (Mason *et al.*, 1992; Arntzen *et al.*, 1994).

1.6.3 Recombinant vaccine vectors

Hitherto, various replicating viral and bacterial vectors have been explored for their potential to generate cell-mediated and antibody responses (Cavanagh, 1985; Flexner *et al.*, 1988). There are advantages in using replication-competent bacteria and viruses, such as *Salmonella*, herpesviruses, adenoviruses and poxviruses over other novel approaches in vaccine development. Upon replication of the vector, amplification of heterologous gene products increases the antigen load. Moreover, such vector-encoded antigens are processed and presented to the immune system in a similar manner to that which occurs during natural infection. Other advantages of recombinant vaccines include the low cost of vaccine preparation and the feasibility of their use in field or clinical situations. Despite these advantages, recombinant vaccine vectors may possess inherent limitations also associated with the use of conventional vaccines, such as causing vaccine-associated complications in immunodeficient recipients as discussed earlier (Section 1.6.2).

1.6.3.1 Recombinant bacterial vectors

Salmonella typhimurium and mycobacteria are examples of bacteria that have been developed as vaccine vectors. Due to the mode of *Salmonella* infection in the gut, this organism has been engineered for delivery of foreign polypeptides of other gut pathogens, such as *Vibrio cholerae* (Tacket *et al.*, 1990) and *Escherichia coli*. Vaccine antigens from *Clostridium tetani* have also been encoded in *Salmonella* in efforts to develop an oral vaccine (Fairweather *et al.*, 1990). Intracellular infection with mycobacteria, leading to the presentation of antigens in association with class I MHC molecules (De Libero *et al.*, 1988), may be useful in eliciting strong cell-mediated immune responses against the encoded vaccine antigen. Indeed, HIV-1 genes expressed by the bacille Calmette-Guerin vaccine strain of mycobacteria induced strong specific cell-mediated and antibody responses for prolonged periods in test animals (Stover *et al.*, 1991). This suggests that antigens encoded by mycobacteria can be presented in association with both class I and class II MHC molecules.

1.6.3.2 Recombinant virus vectors

Adenoviruses, herpesviruses and poxviruses are among those being studied for their potential as vaccine vectors. Early adenovirus vectors, based on human adenovirus type 5 (Ad5), were used to demonstrate the expression of hepatitis B virus surface antigen (HBsAg; Morin *et al.*, 1987). Adenoviruses are potentially important vectors for intranasal (i.n.) immunisation due to the mucosal tropism of the virus, indeed, i.n. immunisation of hamsters with recombinant Ad5 expressing HBsAg has elicited mucosal antibody responses to both vector and HBsAg. In rabbits, others have primed for antibody responses upon intravenous (i.v.) inoculation with a similar construct (Ballay *et al.*, 1985). Human adenoviruses of the Ad4 and Ad7 strains (Top *et al.*, 1971) have also been developed as potential vectors for the expression of the HBsAg and the envelope (env) glycoprotein of HIV; intratracheal immunisation with the recombinant encoding HBsAg generated specific antibodies against the vaccine antigen (Hung *et al.*, 1990).

Potential recombinant herpesvirus vaccine vehicles include HSV-1 and the attenuated varicella-zoster virus strain Oka. Recombinant herpesviruses have been constructed to express HBsAg (Shih *et al.*, 1984) and Epstein-Barr virus membrane glycoprotein (gp) 350/220 (Lowe *et al.*, 1987). The vaccine efficacy of these constructs, however, has not been reported in any detail.

Other recombinant virus vaccines have been constructed from influenza (Li *et al.*, 1993) and pseudorabies viruses (Thomsen *et al.*, 1987). Recently, RNA picornaviruses, such as poliovirus (Evans *et al.*, 1989; Mattion *et al.*, 1994) and mengovirus (Altmeyer *et al.*, 1994), have been developed as potential vaccine vectors. These viruses are incapable of reverse transcription and integration into the host DNA genome due to their replication exclusively in the form of RNA in the cytoplasm of infected cells. However, poxviruses by far are the most studied family of viruses to be developed as candidate vaccine vectors. After the eradication of smallpox and the cessation of smallpox vaccination, VV, the prototype poxvirus, was adapted as a vector (Sam and Dumbell, 1981; Mackett *et al.*,

1982a; Panicali and Paoletti, 1982) where foreign genes were inserted by homologous recombination into the virus genome (reviewed by Moss and Flexner, 1987).

1.7 POXVIRUSES AS VACCINE VECTORS

Poxviruses or *Poxviridae* are large double-stranded DNA viruses with cuboid or prolate virions of 260-450 nm in length (reviewed by Fenner *et al.*, 1989; Moss, 1990a). These viruses multiply in the cytoplasm (Moss, 1990a), display intrageneric genetic recombination and participate in non-genetic reactivation (Christopher *et al.*, 1978). Fenner and Burnet (1957) reviewed these poxviruses in the Poxvirus Subcommittee at the Sixth International Congress for Microbiology (1953). Later, this review formed the basis for subsequent classification of poxviruses. *Poxviridae* are divided into two subfamilies: *Entomopoxvirinae* (poxviruses of insects) and *Chordopoxvirinae* (poxviruses of vertebrates); Table 1.1 shows viruses grouped under the latter category.

Poxvirus recombinants offer several advantages over other replicating virus vectors. These include the ability to accommodate large foreign genes of up to 25 kb while maintaining genome stability (Smith and Moss, 1983). Hence, a recombinant virus can be made to harbour multiple foreign genes of different pathogens, for example surface antigen, gpD and HA of hepatitis B virus, HSV and influenza virus, respectively (Perkus *et al.*, 1985), or be made to express several genes from the same pathogen (Wild *et al.*, 1992), for the development of polyvalent vaccines. Other important advantages of poxvirus vectors include their ability to stimulate both cell-mediated and antibody immunity (McCarthy *et al.*, 1958; Pincus and Flick, 1963) that is relatively long-lasting (Henderson and Fenner, 1994), their heat stability, low cost of preparation, ease of administration and visible proof of vaccination (scars). With replication of poxviruses restricted to the cytoplasm (Moss, 1990a), the risk of viral integration into the host genome is also minimised.

Advances in the development of poxvirus-based vectors have been recently extended to the avian-restricted avipoxviruses, namely fowlpox virus (FPV) and

TABLE 1.1. ***Poxviruses of the vertebrates***

Genus	Species	
	Type species	Other virus species
Orthopoxvirus	Vaccinia	Rabbitpox, variola, cowpox, ectromelia
Parapoxvirus	Orf	Stomatitis papulosa, pseudocowpox
Capripoxvirus	Sheeppox	Goatpox, lumpy skin disease virus
Avipoxvirus	Fowlpox	Canarypox, pigeonpox, turkeypox
Leporipoxvirus	Myxoma	Rabbit fibroma, squirrel fibroma
Suipoxvirus	Swinepox	Nil

Adapted from Buller and Palumbo (1991).

canarypox virus (CPV; Taylor and Paoletti, 1988; Taylor *et al.*, 1992). The development of these avipoxviruses as vaccine vectors will be detailed in Section 1.8.1.

1.7.1 Biology of vaccinia virus

VV is the type species of *Orthopoxvirus*. The VV genome, about 200-kb long (Coupar *et al.*, 1990; Goebel *et al.*, 1990), consists of a linear double-stranded DNA structure and is covalently-linked at the termini (Geshelin and Berns, 1974). The linear genome encodes genes essential for transcription, replication and production of progeny particles independently of host nuclear functions. These termini contain inverted terminal repeats (Garon *et al.*, 1978; Wittek and Moss, 1980), and while genes encoded here are non-essential for replication, they are important for cytopathogenicity which include host range functions (Gillard *et al.*, 1986) and growth factors for viral virulence (Brown *et al.*, 1985; Twardzik *et al.*, 1985; Buller *et al.*, 1988).

Host range. VV has a wide host range that includes humans, primates, rodents and rabbits (Fenner *et al.*, 1989). Infection of permissive cells is followed by cytopathic effects (CPE) or toxic effects, such as cell rounding, granulation and clumping, and progeny virion production. The genetic basis of host restriction of VV was initially ascribed to the left terminus of the genome (Drillien *et al.*, 1981), but later, to host range genes, such as the K1L gene (Rosel *et al.*, 1986) and C7L (Goebel *et al.*, 1990) located at the terminus.

Virus replication. The expression, replication and packaging of the VV genome consists of a series of temporally regulated events. Individual gene products are assigned — based on sensitivity to inhibitors of viral DNA and protein synthesis — to either early, intermediate or late expression (Moss and Filler, 1970; Drillien *et al.*, 1978). Virus-encoded proteins related to VV transcription and the DNA sequences recognised by these specific proteins in early gene expression have been characterised (Moss, 1990b). The entire VV genome is transcribed by the onset of DNA replication.

Early gene products interact with the host during VV replication to ensure adequate levels of precursors for viral DNA synthesis; these interactions mitigate against

the effects of cell cycle control and inhibit host protein synthesis that would lead to cell cytopathy (Moss and Filler, 1970; Drillien *et al.*, 1978). Termination of early transcription occurs about 50 nucleotides downstream from TTTTTNT sequences, in which N can be any nucleotide (Rohrmann and Moss, 1985; Yuen and Moss, 1987). Late gene expression requires different promoter sequences (Keck *et al.*, 1990). RNA transcripts are modified after transcription (Oda and Joklik, 1967; Sebring and Salzman, 1967). Expression of early genes ceases with the onset of late gene expression (Moss, 1990a and 1990b). Proteins made during this stage are largely structural, as well as enzymes essential for the assembly of the structural proteins into progeny virions. Some of these proteins are further modified by glycosylation, phosphorylation, acylation or cleavage reactions before incorporation into virions (Hiller and Weber, 1985; Franke *et al.*, 1989; reviewed by Tower *et al.*, 1988).

Assembly into uniform, spherical, immature particles appears to be sequential and occurs in different stages during virion morphogenesis (de Harvan and Yohn, 1966). During assembly, the DNA genome, enzymes and assorted structural polypeptides enter the particle (Gold and Dales, 1968; reviewed by Dales and Pogo, 1981). Following virion assembly and maturation, the sequence of events involving virus dissemination, such as virus migration to the cell membrane, fusion to the membrane and rupture of the membrane to release virions, has been reported to vary depending on the poxvirus strain and the nature of the host cells (Ichihashi *et al.*, 1971; Dales and Pogo, 1981).

1.7.2 Biology of fowlpox virus

FPV, the type species of *Avipoxvirus*, has chicken as its natural host (reviewed by Tripathy and Cunningham, 1984). FPV is different from other poxviruses in that it cannot be grown in established cells lines (Randall *et al.*, 1964), has the highest lipid content of any poxvirus, contains the largest genome among the poxviruses (Coupar *et al.*, 1990) and does not cause a rapid cessation of cellular DNA synthesis in natural host, for example in chicken epithelial cells (Hanafusa, 1960; Joklik and Becker, 1964; Cheevers *et al.*, 1968; Jungwirth and Launer, 1968; Chan and Hodges, 1973).

Like the VV genome, FPV contains a large and linear double-stranded DNA of approximately 300-kb with covalently-linked terminal hairpins (Coupar *et al.*, 1990). FPV has many regions similar to those found in VV, for example, between the 10.5-kb FPV *Hin*dIII fragment of the FPV genome and the central portion of the VV *Hin*dIII D region (Tartaglia *et al.*, 1990). Furthermore, homology of nucleic acid and amino sequences has also been noted among their thymidine kinase (TK) proteins (Boyle *et al.*, 1987; Binns *et al.*, 1988), DNA polymerases (Binns *et al.*, 1987) and 4b core polypeptides (Binns *et al.*, 1989). However, the FPV TK gene is located in a different region than the TK gene of VV (Boyle *et al.*, 1987; Binns *et al.*, 1988). Similarities between FPV and VV were also noted among many open reading frames (ORF) in FPV and VV (Drillien *et al.*, 1987; Binns *et al.*, 1988; Tomley *et al.*, 1988).

Host range. FPV causes similar cellular pathology in avian cells as other poxviruses (Christopher *et al.*, 1978). However, in contrast to the wide host range of many orthopoxviruses, avipoxviruses are restricted to growth in avian species. While infection of non-permissive mammalian cells with FPV at high multiplicity causes CPE, there is no evidence to suggest that productive replication of viruses occurs in these cells. There has been only one report of the isolation of an avipoxvirus from a mammal: an atypical FPV was isolated from a terminally ill and immunosuppressed rhinoceros in a zoo (Somogyi *et al.*, 1993). Little is known about the fate of avipoxviruses in mammalian cells. A complex family of host range regulatory genes was initially thought to be responsible for host restriction (Perkus *et al.*, 1990). However, Somogyi *et al.* (1993) later showed that the replication 'block' of FPV is due to inefficient expression of late genes and poor maturation of virus particles in mammalian cells.

1.7.3 Strategies for construction of recombinant poxviruses

There are several factors important in the construction of an infectious recombinant VV. First, the inserted foreign DNA should not interfere with expression of essential viral genes. Second, appropriate VV regulatory sequences must be juxtaposed at the 5' end of the foreign gene for transcription by VV-encoded polymerases. Other factors essential for efficient expression of foreign genes are the absence of introns within

the gene and appropriate codon usage, since VV translational machinery has been found to display codon bias or preference (Hruby and Guarino, 1984). Non-essential insertion sites are now well defined in VV. A 9-kb segment at the left terminus of the poxvirus genome has been identified by the isolation of spontaneous mutants (Moss *et al.*, 1981; Panicali *et al.*, 1981; Paez *et al.*, 1985). Other non-essential genes, such as the TK and HA genes, were identified by physical mapping (Weir *et al.*, 1982; Hruby *et al.*, 1983; Shida, 1986). Insertional and deletion mutagenesis have also been employed to locate regions not essential for virus functions (Perkus *et al.*, 1986). While most methods involved attempts to insert genes within non-essential ORF, some investigators have begun to place foreign genes in intergenic sites (Spehner *et al.*, 1990; Fathi *et al.*, 1991; Duncan and Smith, 1992).

Foreign genes were initially inserted empirically into non-essential VV regions (Panicali and Paoletti, 1982), however expression was dependent on a VV promoter being adjacent to sites of insertion. Later, VV promoters and regulatory sequences were engineered proximal to foreign genes. Promoter sequences differ with respect to their temporal regulation, and include the early and late 7.5-kDa promoter (P7.5; Mackett *et al.*, 1984; Cochran *et al.*, 1985), the early *Ava*IH promoter (Perkus *et al.*, 1985; Wachsman *et al.*, 1987) and the late promoter in the 11-kDa gene (Wittek *et al.*, 1984; Cantin *et al.*, 1987). Because of conserved transcriptional machinery in both VV and FPV (Binns *et al.*, 1987 and 1989; Boyle *et al.*, 1987; Boyle and Coupar, 1988a; Prideaux *et al.*, 1990), their transcriptional promoters can function in either virus. The bidirectional early/late and late FPV promoter, P.E/L (Kumar and Boyle, 1990; Prideaux *et al.*, 1990), has been used successfully for gene expression in VV recombinants (Boyle, 1992). Likewise, gene expression driven by the VV H6 promoter (Paoletti and Grady, 1977) in a recombinant avipoxvirus has been successful (Tartaglia *et al.*, 1993).

The isolation of recombinant poxviruses encoding foreign DNA is usually achieved by marker rescue techniques. Unlike other animal and bacterial viruses, such as the baculovirus-insect cell expression system (Summers and Smith, 1987; Luckow and Summers, 1988), co-transfection with purified viral and foreign DNA is not applicable to

the VV expression system. VV DNA is not infectious and virus-associated enzymatic functions are required during initial stages of the replication cycle. Transfected foreign DNA can, therefore, only be 'rescued' using infectious VV particles, the feasibility of this approach first being shown in studies using thermosensitive (Sam and Dumbell, 1981) or deletion (Nakano *et al.*, 1982) mutants. Since then, a variety of marker rescue techniques have been used to isolate recombinant viruses. These include the use of the TK gene (Mackett *et al.*, 1982b; Panicali and Paoletti, 1982), DNA polymerase by virtue of phosphonoacetic-acid resistant marker (Jones and Moss, 1984; Traktman *et al.*, 1984), various temperature-sensitive genetic markers (Condit *et al.*, 1983; Drillien and Spehner, 1983; Ensinger and Rovinsky, 1983; Thompson and Condit, 1986), rifampicin-resistance (Tartaglia and Paoletti, 1985; Baldick and Moss, 1987) and neomycin-resistance (Franke *et al.*, 1985) markers, or using *E. coli* genes for xanthine-guanine phosphoribosyl transferase (*Ecogpt*; Boyle and Coupar, 1988b; Falkner and Moss, 1988) and β-galactosidase (*lac*Z; Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986). Briefly, the process of foreign gene insertion is as follows. The foreign gene is flanked by VV DNA, ranging from 0.1-10 kb in length, which serves as the 'homologous arms' to direct insertion into desired locations within the VV genome. Upon infection with VV and transfection of the engineered insertional plasmid, homologous recombination occurs, and chimeric virus genomes are replicated and packaged into infectious progeny virus.

Other strategies have recently been undertaken in constructing and isolating recombinant viruses. Falkner and Moss (1990) have introduced an approach called 'transient dominant selection' for making recombinant viruses without retaining the selectable marker. While all of the abovementioned strategies have been based on recombination of homologous sequences *in vivo*, the insertion of foreign DNA into the VV genome by direct *in vitro* ligation has also been successful (Merchlinsky and Moss, 1992).

1.7.4 Safety and efficacy of vaccinia viruses as vaccine vectors

The acceptability of VV, or of any virus as a potential vaccine vector, is dependent on many factors. The paramount concerns are those of safety and efficacy (Ada, 1991 and 1994).

Safety. The success of VV as an immunising agent against smallpox points to the potential of poxviruses as effective antigen delivery vehicles (Behbehami, 1983). However, the success of smallpox vaccination was also marred by tragic incidents resulting from vaccine-associated side-effects in some recipients. In immunocompromised individuals, smallpox vaccines cause rare but serious complications, such as eczema vaccinatum (Lane *et al.*, 1969; Lane *et al.*, 1970; Behbehami, 1983) and rare central nervous system disease (de Vries, 1960; Wilson, 1967; Lane *et al.*, 1969; Lane *et al.*, 1970). Vaccination against smallpox has also been reported to cause vaccinal osteomyelitis (Sewall, 1949) and malignant skin tumours, such as melanoma in vaccinated scars, many years later (Marmelzat, 1968). Furthermore, VV has a broad host range (Fenner *et al.*, 1989) which raises the question of species-restriction of the vaccine. Hence, there are still concerns over the reintroduction of poxvirus-based vaccines.

Efficacy. The availability of a highly effective vaccine was a major reason for the success of the Smallpox Eradication Programme (Fenner *et al.*, 1988). Indeed, poxviruses have been shown to elicit long-term immunity in immunised individuals (Henderson and Fenner, 1994). Recently, some volunteers for vaccination with a recombinant VV expressing gp160 of HIV were found to possess antibodies to VV (Cooney *et al.*, 1991), even though they were vaccinated against smallpox many years earlier. In this light, one concern pertaining to the use of VV-based vaccines is whether prior exposure to the immunising vehicle, or to an encoded antigen, will prevent the induction of optimal immune responses upon revaccination. There is evidence for successful revaccination using VV recombinants despite previous exposure to the vector or vaccine antigen (Perkus *et al.*, 1985; Flexner *et al.*, 1988). Conversely, others have found that pre-existing immunity from smallpox vaccination modulated immune responses to a novel antigen expressed by a recombinant VV (Rooney *et al.*, 1988;

Cooney *et al.*, 1991; Etlinger and Altenburger, 1991). These conflicting results may stem from differences in the parameters analysed, however, the use of VV-based vaccines in a large population previously immunised against smallpox requires further evaluation. Moreover, VV infections may expose many endogenous virus-encoded antigens, besides the heterologous vaccine antigen, that will stimulate the immune system. Antigen competition between such proteins may modulate immune responses generated against the vaccine antigen (Andrew *et al.*, 1986).

The efficacy of a vaccine also lies in its ability to generate an appropriate immune response given that pathogens are most effectively controlled by the induction of cell-mediated or antibody responses (Section 1.3.6). Hence, the ability of vaccines to prime for either antibody or cell-mediated immune responses, or both, may not only improve prophylaxis, but also offers the potential to alter the course of disease by vaccine immunotherapy, as previously suggested by Ioannides and Whiteside (1993).

1.7.5 Attenuation of vaccinia viruses

Based on increased understanding of viral virulence factors, efforts have been undertaken to modify VV strains for use as improved immunisation vehicles. Three general approaches have been investigated: adaptation of existing attenuated VV by serial passage in unrelated hosts, selective deletion of specific VV genes and insertion of cytokine genes into the VV genome.

The modified VV Ankara (MVA) is one of the most highly attenuated vaccine strains (Moss, 1994). After more than 570 passages in chicken embryo fibroblasts, the virus was unable to replicate in human and other mammalian cells, and restricted to avian cells. MVA is avirulent in normal and immunosuppressed animals: no significant side-effects have appeared after inoculation of over 120,000 humans, many of whom were at high risk for the conventional smallpox vaccine strains. Later, genetic analysis showed that the attenuation was due to deletions of more than 30-kb DNA, including at least two host range genes, from the genome of MVA (Meyer *et al.*, 1991). The block of MVA replication in non-permissive human cells was found to occur during virion assembly

(Sutter and Moss, 1992). Nevertheless, even during non-productive infections, MVA vectors produce recombinant proteins in a manner similar to wild-type viruses (Sutter and Moss, 1992). Furthermore, the immunogenicity of MVA recombinants in mice resembles that of virulent strains (Moss, 1994), suggesting that MVA may represent a greatly improved VV vector.

Attenuation by specific gene deletions has also been used to modify virulence. Deletions include genes for TK (Buller *et al.*, 1985), a growth factor (Buller *et al.*, 1988), HA (Flexner *et al.*, 1987; Shida *et al.*, 1988), 13.8-K secreted protein (Kotwal *et al.*, 1989), ribonucleotide reductase (Child *et al.*, 1990), env proteins (Rodriguez *et al.*, 1992; Engelstad and Smith, 1993; Wolffe *et al.*, 1993), steroid dehydrogenase (Moore and Smith, 1992), complement control protein (Isaacs *et al.*, 1992) and host range genes (Lee *et al.*, 1992). Subsequently, Paoletti and co-workers have developed and tested a host-range-restricted mutant with 18-gene deletions, called NYVAC (Konishi *et al.*, 1992; Tartaglia *et al.*, 1992). The attenuation of NYVAC was illustrated by the lack of ulceration normally induced on rabbit skin and non-lethality in immunodeficient animals (Tartaglia *et al.*, 1992).

1.7.6 Expression of cytokine genes by vaccinia viruses

Insertion of cytokine genes into the VV genome offers yet another method of decreasing virulence without adversely affecting immunogenicity. IL-2 was the first cytokine gene to be inserted into VV (Flexner *et al.*, 1987; Ramshaw *et al.*, 1987); its expression led to the attenuation of the virus in both normal and immunodeficient mice. Elevated NK cell activity and secretion of IFN-γ by NK and NK-like cells induced by IL-2 was responsible for the attenuation this recombinant VV (Karupiah *et al.*, 1990a, 1990b, 1990c and 1991). Attenuation of the IL-2 recombinant VV in immunocompetent monkeys, as measured by smaller skin lesions, was also noted (Flexner *et al.*, 1990; Ruby *et al.*, 1990). The virulence of VV was also attenuated when IFN-γ (Kohonen-Corish *et al.*, 1990) or TNF-α (Sambhi *et al.*, 1991) was encoded. It is interesting that cytokines decreasing virus virulence are all the Th1 type. On the other hand, encoding a Th2 cytokine, IL-4, was shown to greatly enhance viral pathogenicity by suppressing antiviral

$CD8^+$ CTL activity and down-regulating IL-2, IFN-γ (Th1 cytokines) and IL-12 in the host (Sharma *et al.*, 1995).

VV-expressing IL-5 or IL-6, although not attenuating the virus, selectively stimulated the production of different Ig isotypes against co-expressed vaccine antigens in immunised animals. VV-encoded IL-6 enhanced specific systemic antibody responses of the IgG1 subclass (Ramshaw *et al.*, 1992) and, when delivered at the mucosae, enhanced specific mucosal IgA and IgG antibodies (Ramsay *et al.*, 1994a). Similarly, mucosal infection with recombinant VV encoding IL-5 augmented specific local antibody responses to vaccine antigens (Ramsay and Kohonen-Corish, 1993).

1.8 NOVEL VACCINE VECTORS FOR MAMMALS

Due to the abovementioned problems associated with replicating vectors as vaccines, non-replicating vectors, such as avipoxviruses and nucleic acid vaccine (NAV), have emerged as potential alternatives. In animal studies to date, these vectors have proved to be both safe and effective in eliciting long-term and protective immunity.

1.8.1 Avipoxviruses

Orthopoxviruses have a very broad host range; in contrast, avipoxviruses are restricted to avian species for productive replication (Esposito, 1991). Thus, FPV has been developed as a vaccine vector for poultry (Taylor *et al.*, 1988a and 1990; Edbauer *et al.*, 1990; Nazerian *et al.*, 1992; Heine and Boyle, 1993). FPV infection of mammalian cells is non-productive, i.e. no progeny viruses are produced (Taylor and Paoletti, 1988), however, early gene expression occurs (Somogyi *et al.*, 1993). Indeed, foreign genes engineered under the transcriptional control of an early virus promoter (Taylor *et al.*, 1988b; Cox *et al.*, 1993b), as well as a subset of avipoxvirus-encoded early genes, are appropriately expressed.

These findings have raised the prospect of using avipoxviruses as vaccine vectors in mammalian hosts and, recently, recombinant FPV and CPV (also known as ALVAC) have been studied in non-avian species, such as mice (Cox *et al.*, 1993b), cats (Tartaglia *et al.*, 1993), dogs (Taylor *et al.*, 1991 and 1992) and man (Cadoz *et al.*, 1992). The

expression of the foreign gene products is sufficient to induce both antibody (Taylor *et al.*, 1988b, 1991 and 1992) and cell-mediated immune responses (Cox *et al.*, 1993b) that can afford protective immunity (Taylor *et al.*, 1988b, 1991 and 1992; Tartaglia *et al.*, 1993). Such reponses were elicited for prolonged periods in vaccinated individuals (Taylor *et al.*, 1992; Cox *et al.*, 1993b; Tartaglia *et al.*, 1993).

The abortive nature of avipoxvirus infection in mammalian cells suggests the possible safe use of these viruses as vaccine vectors. Indeed, the safety of ALVAC and its derivative recombinants has recently been demonstrated in immunocompromised recipients (Paoletti *et al.*, 1994). No disseminated infection was observed following intracranial infection with more than 10^7 plaque-forming units (PFU) in newborn mice, young adult mice, genetically deficient nude mice or cyclophosphoamide-treated immunosuppressed mice. In a further study, no significant local or systemic reactogenicity was observed in human volunteers and the recombinant ALVAC was well-tolerated (Cadoz *et al.*, 1992).

1.8.2 Nucleic acid vaccines

NAV technology had its birth with the demonstration that large quantities (> 100 μg) of DNA expression vectors, when injected into mice intramuscularly (i.m.), were taken up by muscle cells (Wolff *et al.*, 1990) that are thought to persistently express the encoded genes (Wolff *et al.*, 1992). Muscle may be particularly suited for harbouring and expressing NAV; its structural features allow cell regeneration after damage due to injection, while the longevity of muscle cells may account for the prolonged expression of the encoded gene product *in vivo* (Wolff *et al.*, 1992). Subsequently, other investigators reported gene expression after direct injection of DNA into heart and skeletal muscle cells of rats (Lin *et al.*, 1990; Acsadi *et al.*, 1991b; Kitsis *et al.*, 1991) and dogs (Vonharsdorf *et al.*, 1993), and in the diaphragm (Davis and Jasmin, 1993) and neurons (Sahenk *et al.*, 1993). Expression was also found following i.m. injection in other animals, including fish (Hansen *et al.*, 1991), dogs (Vonharsdorf *et al.*, 1993), rabbits (Gal *et al.*, 1993; Agadjanyan *et al.*, 1994), cattle (Cox *et al.*, 1993a) and primates (Jiao *et al.*, 1992; Donnelly *et al.*, 1994).

Other routes of NAV administration have also been attempted, including intraperitoneal (i.p.), i.n., subcutaneous (Fynan *et al.*, 1993) and i.v. injection (Fynan *et al.*, 1993; Zhu *et al.*, 1993). An i.v. inoculation of chloramphenicol acetyltransferase-encoding NAV, coated with liposomes, transfected virtually all tissues, including the lungs, spleen, lymph nodes and bone marrow of recipient mice (Zhu *et al.*, 1993). Another mode of NAV delivery that has proven highly successful is the particle bombardment or 'gene-gun' method (Tang *et al.*, 1992; Fynan *et al.*, 1993; Haynes *et al.*, 1994). Gene-gun delivery of NAV to the epidermis offers a direct and intracellular delivery of very small amounts (< 1 μg), as well as a practical and easy delivery site. This method has been shown to induce protective immunity against lethal challenge with influenza virus (Fynan *et al.*, 1993).

The elicitation of specific-antibodies by NAV was first described in mice inoculated with vectors encoding human growth hormone by the gene-gun method (Tang *et al.*, 1992). However, the vaccine potential of NAV was first explored by Ulmer *et al.* (1993), who reported that mice immunised i.m. with NAV expressing a conserved internal protein of influenza virus, the NP, mounted both NP-specific CTL and antibody responses, and were protected against subsequent challenge with a heterologous strain of influenza virus. Protection against a homologous strain of influenza virus was also obtained after immunisation of chicken with HA-encoding NAV (Robinson *et al.*, 1993). Since these reports, direct injection of NAV has been extended to include numerous other genes, including genes encoding env of HIV (Wang *et al.*, 1993) and circumsporozoite protein of *Plasmodium yoelii* (Sedegah *et al.*, 1994). Mice given the NAV-HIV env recombinant i.m. developed neutralising antibodies against HIV while $CD8^+$ T lymphocyte-dependent protection against malaria was attained in mice immunised with NAV expressing the circumsporozoite antigen.

Immunisation with DNA has been shown to be a more efficient method for delivery of antigen into muscle than other vectors, such as adenoviruses and retroviruses (Davis *et al.*, 1993). Injection of NAV results in the generation of long term immunity (Ulmer *et al.*, 1993; Agadjanyan *et al.*, 1994; Sedegah *et al.*, 1994) and this may be due to

prolonged expression of encoded genes, by long-lived muscle cells harbouring the NAV, for many months after immunisation (Wolff *et al.*, 1992). In addition, the advantages of NAV for revaccination have been amply illustrated in vaccinated individuals (Fynan *et al.*, 1993; Ulmer *et al.*, 1993). A most desirable feature is that NAV may represent a safe approach to vaccination. Despite widespread use, there are, as yet, no reports of integration of injected plasmids into the genome of mammalian cells (Wolff *et al.*, 1992). Furthermore, immunisation with NAV has not been shown to elicit anti-DNA responses (Jiao *et al.*, 1992) that could lead to autoimmunity in vaccinated individuals. Nevertheless, these aspects of NAV vaccination should continue to be monitored in experimental studies.

1.9 OBJECTIVES AND SCOPE OF THIS THESIS

The advent of non-replicating vectors has improved prospects for the development of safe and effective vaccines against a variety of diseases. Moreover, the effectiveness of vaccines can be further improved by their ability to induce appropriate immune responses. Cytokines play critical roles in the development of immune responses. The aims of this work are to study the potential of cytokines expressed in recombinant viruses to modify antiviral immune responses and to investigate the use of different vaccine strategies in attempts to generate augment immune responses against encoded vaccine antigen.

In **Chapter 2**, the construction of recombinant FPV expressing the HA gene of influenza virus A/Puerto Rico/8/34 (A/PR/8) together with genes for murine IL-6 or IFN-γ is described. The use of these recombinants and the influence of the FPV-encoded cytokines in generating both systemic and mucosal immune responses against HA are examined in **Chapter 3**. The safety of recombinant FPV, their long-term expression of encoded genes, and their ability to induce immune responses in animals will also be studied. The efficacy of these constructs in affording protection against lethal challenge with influenza virus is also documented.

In **Chapter 4**, studies of the use of NAV immunisation in inducing protective immune responses are reported. The combination of this strategy with that of

recombinant FPV, each expressing the common HA antigen, to generate greatly enhanced immune responses will also be investigated. Both systemic and mucosal antibody responses after NAV immunisation i.m. and boosting with recombinant FPV at the respective site are studied. The effects of FPV-encoded cytokines on boosting of immune responses are also described.

In **Chapter 5**, the construction of a recombinant VV expressing murine IL-7 and the immune regulatory effects of this cytokine during antiviral immunity are recorded. The functional roles of a number of cytokines in the immune responses have been previously studied successfully using similar approaches (Ramshaw *et al.*, 1992; Ramsay and Kohonen-Corish, 1993; Ramsay *et al.*, 1994a and 1994b).

CHAPTER 2

Construction and in vitro *testing of*

recombinant fowlpox viruses expressing cytokine genes

2.1 INTRODUCTION

VV is a highly immunogenic virus capable of stimulating long-term cell-mediated and antibody immunity (McCarthy *et al.*, 1958; Pincus and Flick, 1963; Henderson and Fenner, 1994). The availability of a highly effective vaccine was a major reason for the success of the Smallpox Eradication Programme (Fenner *et al.*, 1988). However due to rare but serious vaccine-induced complications occurring in immunocompromised individuals (Lane *et al.*, 1969; Lane *et al.*, 1970; Behbehami, 1983), there are still concerns over the use of VV in humans. The acceptability of a virus vaccine depends on the paramount concerns of safety and efficacy (Ada, 1991 and 1994).

Host-restricted poxviruses, such as the avian species-restricted avipoxvirus, have been developed as alternative vaccine vehicles. Avipoxviruses, for example FPV, have been used as vaccine vectors for immunogens of fowl pathogens in poultry (Taylor *et al.*, 1988a and 1990; Edbauer *et al.*, 1990; Nazerian *et al.*, 1992; Heine and Boyle, 1993). In mammalian cells, these viruses cause abortive infections (Taylor and Paoletti, 1988; Somogyi *et al.*, 1993) and thus cannot produce mature infectious virions. Although unable to replicate in mammals, avipoxviruses nevertheless can express appropriately engineered foreign genes under the transcriptional control of early poxvirus promoters (Taylor *et al.*, 1988b; Cox *et al.*, 1993b). CPV have been shown to be safe and effective vectors capable of eliciting prolonged immunity in mammals (Taylor *et al.*, 1991 and 1992; Cadoz *et al.*, 1992; Cox *et al.*, 1993b; Tartaglia *et al.*, 1993).

Cytokine gene expression has been used to decrease the virulence of VV vectors without adversely affecting the immunogenicity of the virus. For example, attenuation of VV expressing mouse or human IL-2 in immunodeficient athymic nude mice has been reported by Ramshaw *et al.* (1987) and Flexner *et al.* (1987), and in immunocompetent monkeys by Flexner *et al.* (1990) and Ruby *et al.* (1990). The growth of recombinant VV encoding IFN-γ (Kohonen-Corish *et al.*, 1990) or TNF-α (Sambhi *et al.*, 1991) was also attenuated to the extent that infection could be rapidly resolved by severely immunodeficient mice. Further, local expression of cytokines during VV replication has been used to selectively manipulate the immune response generated against co-expressed

heterologous antigens (Ramshaw *et al.*, 1992). Such approaches have allowed studies to be undertaken into the immunobiology of several cytokines. For example, IL-6 expression by recombinant VV at mucosae has revealed the pivotal role of this cytokine in the development of mucosal antibody responses (Ramsay *et al.*, 1994a).

In this chapter, the construction of recombinant FPV vectors expressing the HA gene of influenza virus strain A/PR/8 together with murine cytokines IL-6 or IFN-γ is described.

2.2 MATERIALS AND METHODS

2.2.1 Culture medium

F15 medium is made of Eagles Minimum Essential Medium (Cytosystems, Castle Hill, NSW, Australia) supplemented with $NaHCO_3$ (2.2 g/l), L-glutamine (216 mg/l), fetal calf serum [5% (v/v); FCS; Flow Laboratories, North Ryde, Australia], PSN [penicillin (30 mg/l), streptomycin sulphate (50 mg/l) and neomycin sulphate (50 mg/l)] and 2-mercaptoethanol (50 μM).

For culturing hybridomas and cytokine-dependent cells, Mixed Lymphocyte Medium (MLC) was used. MLC is made of DMEM (GIBCO BRL, Life Technologies, MD, USA) supplemented with glucose (4 g/l), folic acid (6 mg/ml), L-asparagine (36 mg/l), L-arginine (116 mg/l), sodium pyruvate (1 mM), L-glutamine (216 mg/l), HEPES (10 mM), 2-mercaptoethanol (50 μM), FCS [10% (v/v)] and PSN. Supernatant [10% (v/v)] from con A-stimulated spleen cells was added to MLC during growth of cytokine-dependent cell lines as a source of growth factors.

2.2.2 Cell lines and hybridomas

Human osteosarcoma 143B cells (Rhim *et al.*, 1975) and CV-1, a cell line derived from African green monkey (Jensen *et al.*, 1964), were grown in F15 medium. Chicken embryo skin (CES) cells, a primary epithelial cell line, were obtained from 13-day old embryos as described in Section 2.2.3 and maintained in F15 medium, and used before three passages.

The IL-6-dependent cell line, B9 (Aarden *et al.*, 1987), and the rat anti-murine IFN-γ antibody secreting hybridoma, R4.6A2 (Spitalny and Havell, 1984), were grown in MLC.

2.2.3 Preparation of primary CES cells

Primary CES cells were prepared using the method of Silim *et al.* (1982). Specific pathogen-free (SPF) eggs were candled after 13 days of incubation, and embryos from these eggs were harvested and washed extensively with Puck's saline A [phenol red

(0.5 mM), NaCl (140 mM), KCl (5.4 mM), glucose (5.6 mM) and $NaHCO_3$ (4 mM), pH 7.2-7.4]. After washing, the embryos were transferred to Puck's saline A (25 ml/embryo) containing collagenase (100 µg/ml; Sigma Chemical Co., MO, USA) and $CaCl_2$ (5 mM). The mixtures were incubated at 37°C, with gentle and constant stirring, for 30 min. The embryos were then removed leaving the skin in solution and digestion was allowed to proceed for another 10 min at 37°C. The cell suspension was then filtered through 4 layers of sterile gauze to remove large debris and cell clumps, and pelleted by centrifugation. The cell pellet was resuspended in F15 medium and seeded in plastic roller bottles (850 cm^2; Corning, NY, USA) at 150 x 10^6 cells in 200 ml of F15 medium.

2.2.4 Viruses

FPV-M3, a tissue culture passage strain derived from the mild vaccine strain, FPV-Web (Arthur Webster Pty Ltd, Northmead, Australia), was grown in CES cell monolayers as described (Boyle *et al.*, 1987). VV-HA (TK^+), VV-HA-IL6 and VV-HA-IFNγ used here have been made and described elsewhere by Coupar *et al.* (1988), Ramshaw *et al.* (1992) and Kohonen-Corish *et al.* (1990), respectively.

FPV and VV stocks were grown in acid-washed 2-l Schott or plastic rollers (850 cm^2; Corning) on CES and CV-1 monolayers, respectively. These monolayers were infected at a multiplicity of infection (moi) of 0.01 (i.e. 10^{-2} PFU/cell). FPV stocks were harvested after 5-7 days while VV stocks were collected at 48 h after infection. The titres of stock viruses were determined by plaque assay and stored in 1-ml aliquots at -75°C. VV stocks were sonicated twice at 50 W for 5 sec using a Branson Sonifier (Branson Sonic Power Co., CT, USA), whereas FPV stocks were trypsinised [0.5% (w/v); Sigma] for 30 min at 37°C before neutralisation with FCS [50% (v/v)]. Viruses were diluted in gelatin-saline to appropriate titres (usually 5 x 10^7 PFU/ml) before use.

2.2.5 DNA manipulation, polymerase chain reactions (PCR), sequencing and Southern blotting

Molecular cloning, restriction enzyme (RE) digestions and other recombinant DNA techniques were performed using conventional procedures (Sambrook *et al.*, 1989).

RE and other molecular biology reagents were purchased from Boehringer Mannheim, GmbH, Germany (BM) and Bethesda Research Laboratory, MD, USA (BRL).

PCR reactions for producing DNA fragments for subcloning were performed with 1 ng of CsCl-purified plasmid {total reaction volume of 18 µl containing SuperTaq (0.2 units; P.H. Stehelin & CIE AG, Basel), Tris (10 mM; pH 9.0), KCl (50 mM), gelatin [0.01% (v/v)], $MgCl_2$ (1.5 mM), Triton X-100 [0.1% (w/v)], dNTPs (G, A, T and C, each at 2.5 mM) and primers (each at 5 µM)} using a capillary FTS-Thermal Sequencer (Corbett Research, Australia). The reactions were carried out as follows — 95°C, 5 min (1 cycle): 95°C, 5 sec; 53°C, 2 sec; 72°C, 1 min (25 cycles): 72°C, 5 min (1 cycle). PCR products were then purified by phenol extraction after agarose gel purification and digested with appropriate REs before use for subcloning into phagemid pTZ19U (Mead *et al.*, 1986). PCR reaction of FPV DNA was performed using an AmpliTaq kit (Perkin-Elmer Cetus, NJ, USA) using PCR machine HYBAID (Hook and Tucker Instruments Ltd., UK) as outlined by Heine and Boyle (1993) — 95°C, 1 min; 60°C, 2 min; 72°C, 5 min (30 cycles), with the elongation time in each cycle extended by 20 sec. PCR products were then analysed by agarose gel [1-1.5% (w/v)] electrophoresis in TBE buffer, pH 8.3.

For sequencing, single-stranded DNA templates were prepared from recombinant phagemid pTZ19U maintained in *E. coli* DH11S (Lin and Mattson, 1991) and sequenced by the dideoxy-chain termination method of Sanger *et al.* (1977) using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., CA, USA). Analysis of DNA sequences was performed using SeqEd version 1.0 (Applied Biosystems, Inc.).

For Southern blotting, DNA fragments were transferred onto a nylon membrane (GeneScreen Plus, DuPont, MA, USA), by capillary blotting under alkaline conditions (0.4 M NaOH), after agarose gel electrophoresis. The Southern blot was then blocked with 1.0% (w/v) skim milk in hybridisation buffer {SSPE (2X), SDS [7% (w/v)], herring testes DNA (0.5 mg/ml; Sigma)} for 2-6 h at 65°C. The blot was probed with

heat-denatured ^{32}P-labelled DNA probe in hybridisation buffer with gentle mixing at 68°C (HYBAID Mini Oven MKII, Hybaid Ltd., UK). After hybridisation for 14-20 h, unbound DNA probe was removed under high stringency (68°C) by washing in SSC (2X) and SDS [0.1% (w/v)], and then in SSC (0.2X) and SDS [0.1% (w/v)] for 20-30 min each before the membrane was air-dried and used for autoradiography.

2.2.6 Primers

Primers PHA-1 and PHA-2 (Appendix I) complementary to the 5' and 3' ends, respectively, of the ORF of the HA gene of influenza virus A/PR/8 were designed with *Bam*HI and *Bgl*II restriction sites for PCR fragment subcloning. PHA-2 also contains two overlapping transcriptional termination signals, TTTTTTTT (Boyle, 1992), and an in-frame translational stop codon for termination of expression at the 3' end.

PIL6-1 and PIL6-2 (Appendix I) containing transcriptional and translational stop signals, and *Bam*HI restriction sites for subcloning of PCR fragments, were used to prepare murine IL-6 DNA for subcloning. For subcloning the murine IFN-γ gene, primers described in Appendix I, with similar features to the primers for IL-6, were used.

PCR primers P330 and P348 (Appendices I and II), homologous to the FPV TK gene and the *Nco*I site downstream of the TK gene (Heine and Boyle, 1993), respectively, were used for PCR analysis of recombinant FPV genome arrangements.

2.2.7 Construction of recombinant viruses

FPV recombinants were constructed as described elsewhere (Boyle and Coupar, 1988a) using the co-expressed *Ecogpt* gene for recombinant virus amplification and selection (Boyle and Coupar, 1988b). CES were infected with FPV-M3 at moi of 0.01 for 1 h and these monolayers were then transfected with 10 μg of plasmid in 0.2 mg DEAE-dextran (molecular weight 2 x 10^6; Pharmacia, NJ, USA)/ml. After 16-24 h, monolayers were washed and incubated for 6-7 days in F15 medium containing MXHAT [mycophenolic acid (2.5 μg/ml), xanthine (250 μg/ml), hypoxanthine (0.1 mM), aminopterine (0.4 μM) and thymidine (30 μM)].

After three cycles of growth under MXHAT selection, recombinant viruses (containing a *lac*Z gene) were purified by plaquing under non-selective conditions with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (0.3 mg/ml; BRL) in F15 medium. Two or three cycles of purification of blue-plaque recombinant viruses were performed until the virus population was homogeneous. DNA from recombinant viruses was analysed by PCR using primers P330 and P348 (Appendices I and II), as described previously (Heine and Boyle, 1993), to confirm both the insertion of heterologous DNA and the absence of wild-type FPV-M3 in recombinant FPV stocks.

2.2.8 Preparation of FPV DNA

The method described by Heine and Boyle (1993) was used for preparing FPV DNA. Aliquots of virus stocks, 100 µl, were digested with proteinase K (500 µg/ml; BM) in Tris (10 mM; pH 7.6), 2-mercaptoethanol (50 mM), NaCl (100 mM), EDTA (10 mM), sarkosyl [1% (w/v)] and sucrose [26% (w/v)] for 1 h at 58°C, and the lysate was then extracted with phenol/chloroform (50%:50%, v/v). DNA was precipitated with ethanol, digested with RNase (20 mg/ml) and denatured at 100°C for 10 min before use.

2.2.9 Cytokine assays

Supernatants from cell cultures infected with recombinant virus (moi 1) were passed through 0.1 µm filters to remove virus particles prior to assay. The presence of IL-6 was detected by [^{3}H]-thymidine incorporation (78 Ci/mmole; ICN Radiochemicals, CA, USA) using the IL-6-dependent cell line, B9. Cytokine-dependent cells (5×10^3 cells/well), in flat-bottomed 96-well plates (Nunc, Roskilde, Denmark), were cultured for 48 h at 37°C in MLC containing serially diluted supernatant from infected monolayers. Wells were pulsed with [^{3}H]-thymidine (1 µCi) during the last 4-6 h of culture. Cells were then harvested onto filter mats using a Skatron 96 well harvester (Pharmacia). Scintillation fluid (LKB Scintillation Products, Leics, UK) was added and [^{3}H]-thymidine uptake was measured in a Pharmacia liquid scintillation counter.

IFN-γ in supernatants was detected by indirect ELISA, as described elsewhere (Sheehan *et al.*, 1989; Carpenter *et al.*, 1994), using the purified rat anti-murine IFN-γ

monoclonal antibody, R4.6A2, as the capture antibody and a polyclonal rabbit anti-murine IFN-γ (Silenus Laboratories Pty. Ltd., Australia) as the secondary antibody. ELISA plates (Titertek, Flow Laboratories, The Netherlands) were coated with the capture antibody [1:5000 (v/v)] in carbonate buffer [$NaHCO_3$ (35 mM) and Na_2CO_3 (15 mM), pH 9.6] at 4°C overnight. These plates were then blocked for 2 h at room temperature with skim milk [5% (w/v) in PBS]. Two-fold serially diluted samples were added to the wells and incubated at 37°C for 2 h. After removal of samples by washing with PBS-Tween-20 [0.2% (v/v); Sigma], the secondary antibody [1:1000 (v/v)] was added and incubated at 37°C for 2 h. The cytokine-antibody complexes were detected using a polyclonal anti-mouse IFN-γ antisera and alkaline phosphatase-conjugated anti-rabbit Ig (Silenus). The assay was then developed at room temperature by application of substrate (*p*-nitrophenyl phosphate, disodium, 5 mg/ml; Sigma) in alkaline buffer (2-amino-2-methyl-1-propanol buffer; 1.5 M; pH 10.3; Sigma). The enzymatic product was read at 430 nm with a THERMOmax microplate reader (Molecular Devices, CA, USA). ELISA results were analysed using SOFTmax programme version 2.0.1 (Molecular Devices).

Cytokine titres were determined by comparison of the dilution giving half maximal proliferation of cytokine-dependent cells, or in the case of ELISAs, by comparison of optical density with that of a concurrently performed appropriate standard recombinant murine cytokine.

2.2.10 Immunofluorescence assays

143B monolayers, grown for 1 day on acid-washed glass cover slips, were infected with virus at a moi of 1 for 24 h at 37°C. Monolayers were then washed with pre-chilled PBS before incubation with anti-HA antiserum [1:100 (v/v); raised in rabbits] for 30 min at room temperature. After washing, membrane-bound antibodies were detected by FITC-conjugated anti-rabbit Ig antibody (Silenus). Immunofluorescence was examined by ultraviolet microscopy.

2.2.11 Statistical analysis

Data were expressed as means ± standard error of the mean (SEM) or standard deviation (SD) and a two-tailed Student's *t* test was used to determine the significance of the differences between experimental and control groups. The Student's *t* tests were performed using InStat for MacIntosh version 2.1. Statistical significance was set at $p < 0.05$ for all comparisons. Each experiment was repeated at least twice and accepted as valid only when similar results were obtained in at least 2 experiments.

2.3 RESULTS

2.3.1 Construction of FPV plasmid insertion vectors

A novel FPV insertion plasmid, pLRB100, was derived from pAF09 (Heine and Boyle, 1993) for the construction of recombinant FPV expressing the influenza virus A/PR/8 HA and murine cytokine genes (Fig. 2.1). Plasmid pLRB100 contains the HA gene inserted as a 1.8 kb *Bgl*II fragment proximal to the early promoter of a FPV bidirectional promoter, P.E/L (Kumar and Boyle, 1990) and an additional copy of the P.E/L promoter at the 3' end of the HA gene. This additional promoter was obtained from pAF03 (kindly provided by Dr. D.B. Boyle, Australian Animal Health Laboratory, Geelong, Australia) into which the HA gene, derived from pJZ102 (Young *et al.*, 1983) by PCR, was initially cloned (Fig. 2.2). In pLRB100, the ATG initiation codon of HA is preceded by four codons in alignment with the ATG initiation codon of the P.E/L promoter to ensure in-frame translation. In addition, pLRB100 contains a *Bam*HI site in front of the 3'-end of the second P.E/L promoter for further insertion of cytokine genes.

Plasmids pLRB120 and pLRB140 contain a copy of PCR-derived IL-6 and IFN-γ genes, respectively, at the *Bam*HI site of pLRB100 (Fig. 2.3). Insertion of a PCR-derived HA gene directly into pAF09 gave rise to pLRB130 (Fig. 2.1). The initiation codon of each heterologous gene is also preceded by four codons in alignment with the initiation codon of the P.E/L promoter. Nucleotide sequences of the PCR-derived HA, IL-6 and IFN-γ genes were confirmed by sequencing.

2.3.2 Expression of HA and cytokine genes by recombinant FPVs *in vitro*

Transfection of FPV-M3-infected CES cells with plasmid insertion vectors pLRB130, pLRB120 and pLRB140 resulted in isolation of recombinants FPV-HA, FPV-HA-IL6 and FPV-HA-IFNγ, respectively. The genomic arrangements of the recombinant FPV are illustrated in Fig. 2.4. The purity of recombinant FPV virus stocks was confirmed by PCR analysis with primers P330 and P348: PCR of FPV-M3 DNA gave rise to a ~ 0.4-kb fragment unlike the other recombinant FPV (Fig. 2.5). PCR of recombinant DNA, in theory, should produce fragments of > 3 kb, however, these large

FIGURE 2.1

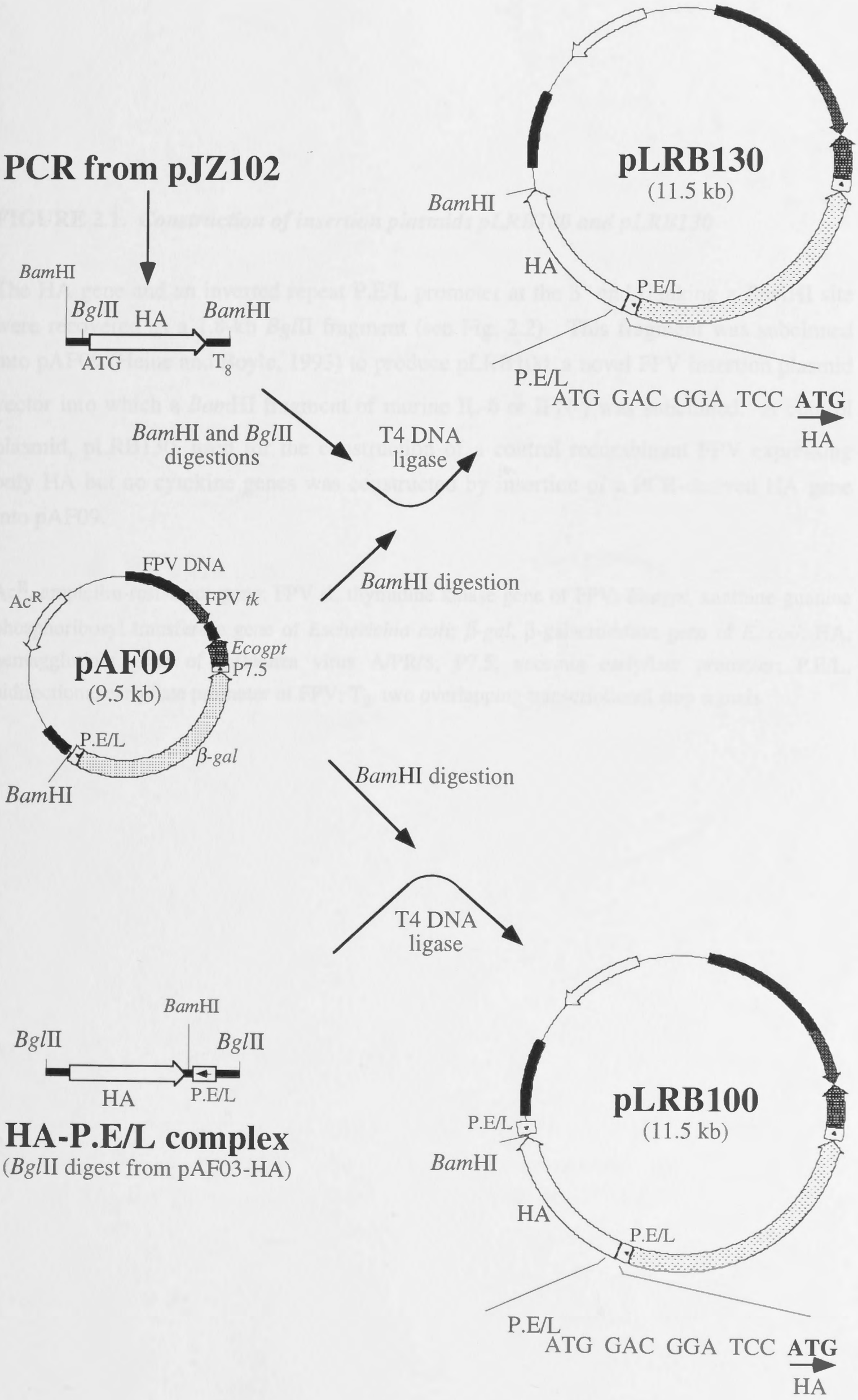

PCR from pJZ102
BamHI
BglII
HA
BamHI
ATG
T8
BamHI and BglII digestions
T4 DNA ligase
pLRB130
(11.5 kb)
BamHI
HA
P.E/L
P.E/L
ATG GAC GGA TCC ATG
HA
FPV DNA
AcR
FPV tk
Ecogpt
P7.5
pAF09
(9.5 kb)
P.E/L
β-gal
BamHI
BamHI digestion
BamHI digestion
T4 DNA ligase
BamHI
BglII
BglII
HA
P.E/L
HA-P.E/L complex
(BglII digest from pAF03-HA)
pLRB100
(11.5 kb)
P.E/L
BamHI
HA
P.E/L
P.E/L
ATG GAC GGA TCC ATG
HA

FIGURE 2.1. ***Construction of insertion plasmids pLRB100 and pLRB130***

The HA gene and an inverted repeat P.E/L promoter at the 3' end flanking a *Bam*HI site were recovered as a 1.8-kb *Bgl*II fragment (see Fig. 2.2). This fragment was subcloned into pAF09 (Heine and Boyle, 1993) to produce pLRB100, a novel FPV insertion plasmid vector into which a *Bam*HI fragment of murine IL-6 or IFN-γ was subcloned. A control plasmid, pLRB130, used for the construction of a control recombinant FPV expressing only HA but no cytokine genes was constructed by insertion of a PCR-derived HA gene into pAF09.

Ac^R, ampicilin-resistance gene; FPV *tk*, thymidine kinase gene of FPV; *Ecogpt*, xanthine-guanine phosphoribosyl transferase gene of *Escherichia coli*; *β-gal*, β-galactosidase gene of *E. coli*; HA, hemagglutinin gene of influenza virus A/PR/8; P7.5, vaccinia early/late promoter; P.E/L, bidirectional early/late promoter of FPV; T_8, two overlapping transcriptional stop signals

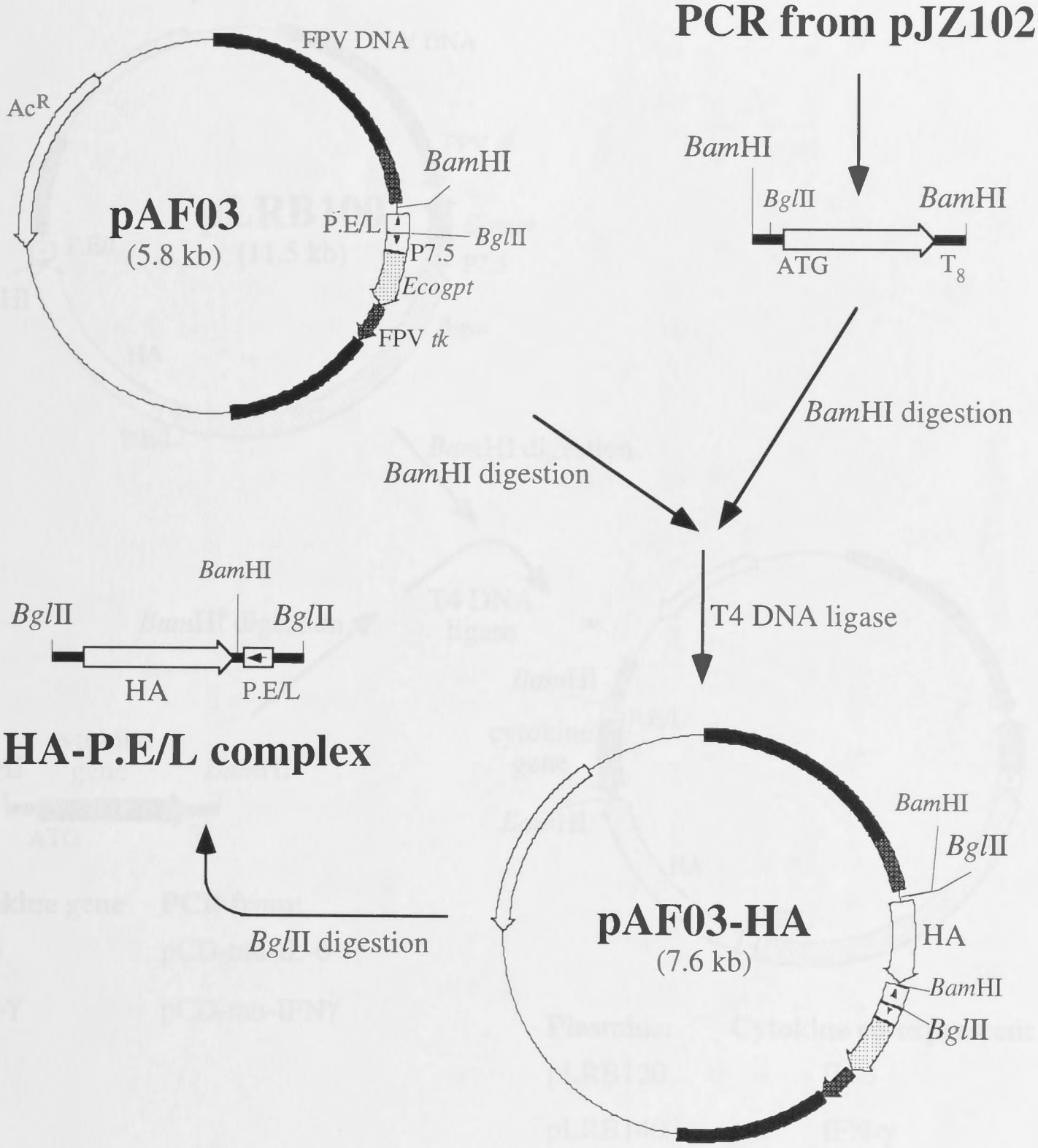
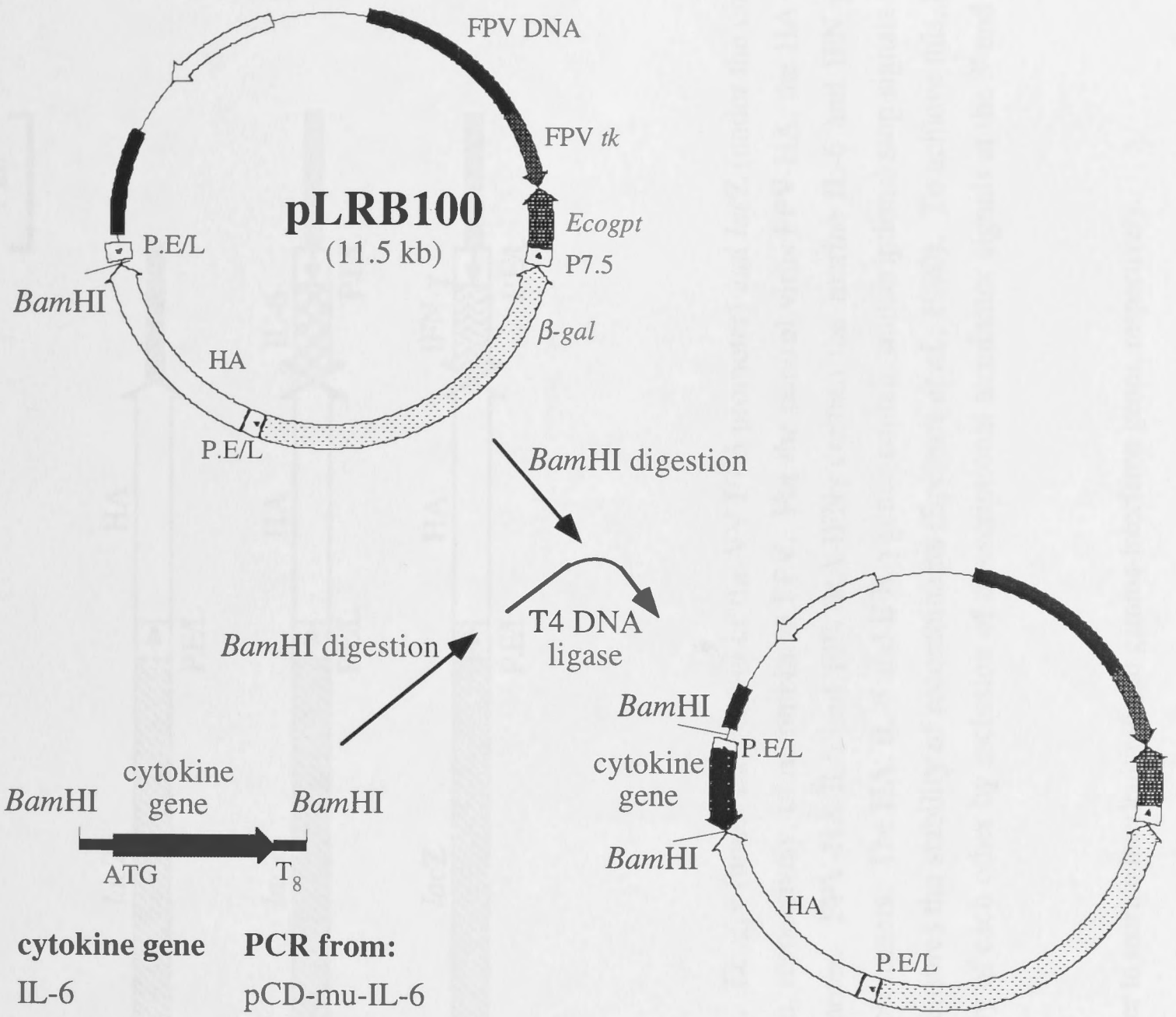


FIGURE 2.2. ***Construction of plasmid pAF03-HA***

The HA gene was amplified by PCR from pJZ102 (Young *et al.*, 1983) using PCR primers PHA-1 and PHA-2, and subcloned into pAF03 (kindly provided by Dr. B.B. Boyle, AAHL). The HA gene and an inverted repeat P.E/L promoter at the 3' end flanking a *Bam*HI site were recovered as a 1.8-kb *Bgl*II fragment.

Abbreviations are as for Fig. 2.1.



cytokine gene	PCR from:
IL-6	pCD-mu-IL-6
IFN-γ	pCD-mu-IFNγ

Plasmids:	Cytokine co-expressed:
pLRB120	IL-6
pLRB140	IFN-γ

FIGURE 2.3. ***Construction of insertion plasmids pLRB120 and pLRB140***

Plasmid pLRB120 contains a copy of the IL-6 gene, derived from pCD-muIL6 (Chiu *et al.*, 1988) by PCR using primers PIL6-1 and PIL6-2, at the *Bam*HI site of pLRB100. Plasmid pLRB140 was constructed similarly with the IFN-γ gene derived from pCD-muIFNγ (Gray and Goeddel, 1983) by PCR.

Abbreviations are as for Fig. 2.1.

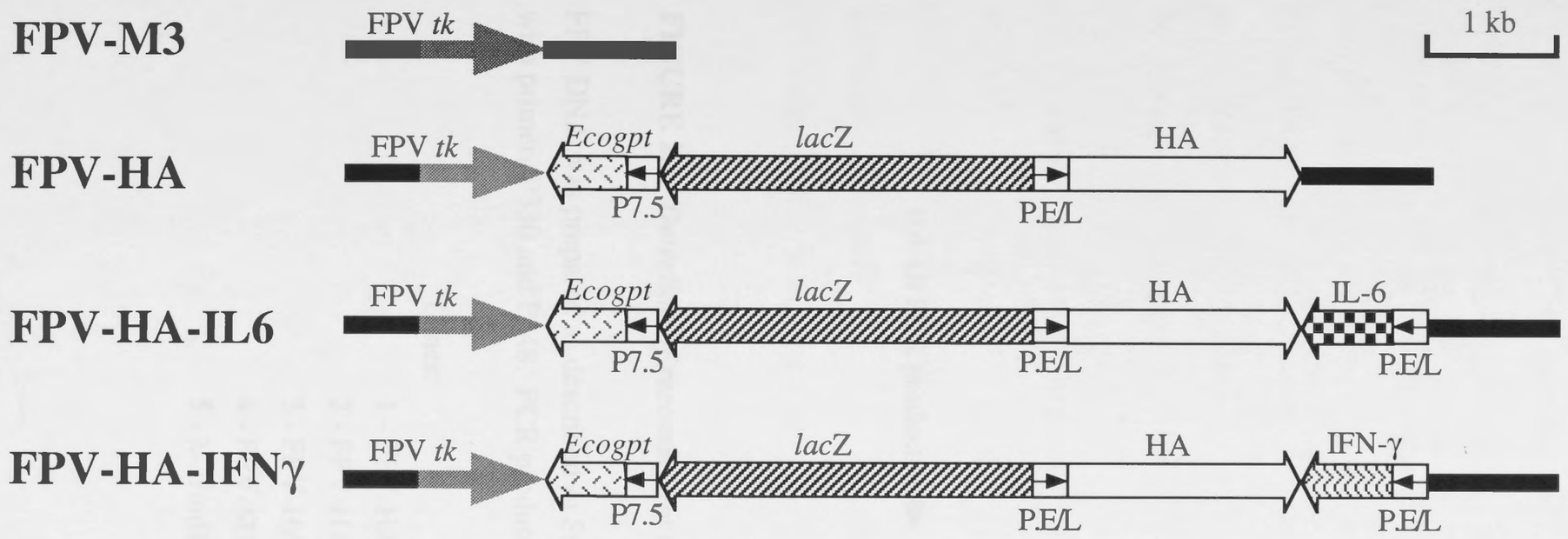


FIGURE 2.4. ***Genomic arrangements of FPV***

Foreign genes were inserted at a single site downstream of the *tk* gene. *Ecogpt* (under the control of the VV P7.5 promoter) and *lac*Z (under the control of the late P.E/L promoter) genes were used for selection and purification, respectively, of recombinant FPV. For the control virus FPV-HA, the HA gene of influenza virus was subcloned proximal to the early/late P.E/L promoter. FPV-HA-IL6 and FPV-HA-IFNγ contain the murine IL-6 and IFN-γ genes, respectively, each under the transcriptional control of early/late P.E/L promoters. The HA, IL-6 and IFN-γ genes contain transcriptional stop signals at the 3' ends. The use of inverted repeats of the promoter, in this case P.E/L, ensures the stability of recombinants (Spehner *et al.*, 1990). To achieve this, HA and cytokine genes were expressed in opposite directions and independently of each other by inclusion of transcriptional terminator signals at the 3' end of each gene.

Abbreviations are as for Fig. 2.1, except for the following: IL-6 and IFN-γ refer to murine interleukin-6 and gamma-interferon genes, respectively.

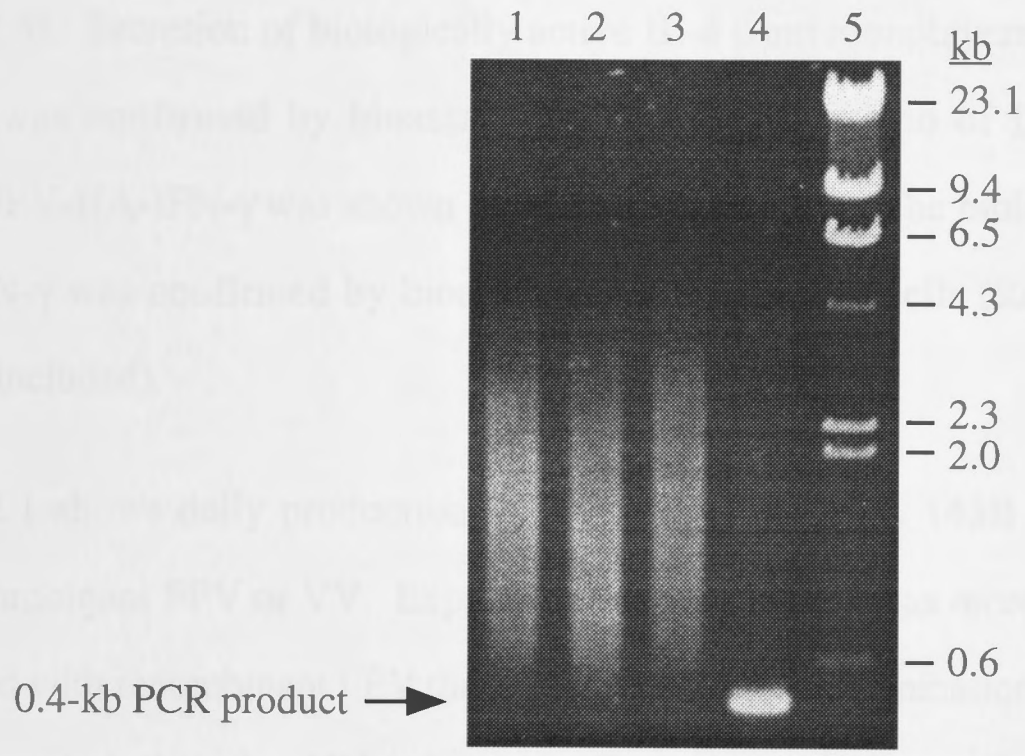


FIGURE 2.5. ***Detection of recombinant and wild-type FPV by PCR***

FPV DNA was prepared as described in Section 2.2.8 and 10 ng of DNA was used for PCR with primers P330 and P348. PCR products were analysed on a 1.0 % (w/v) agarose gel.

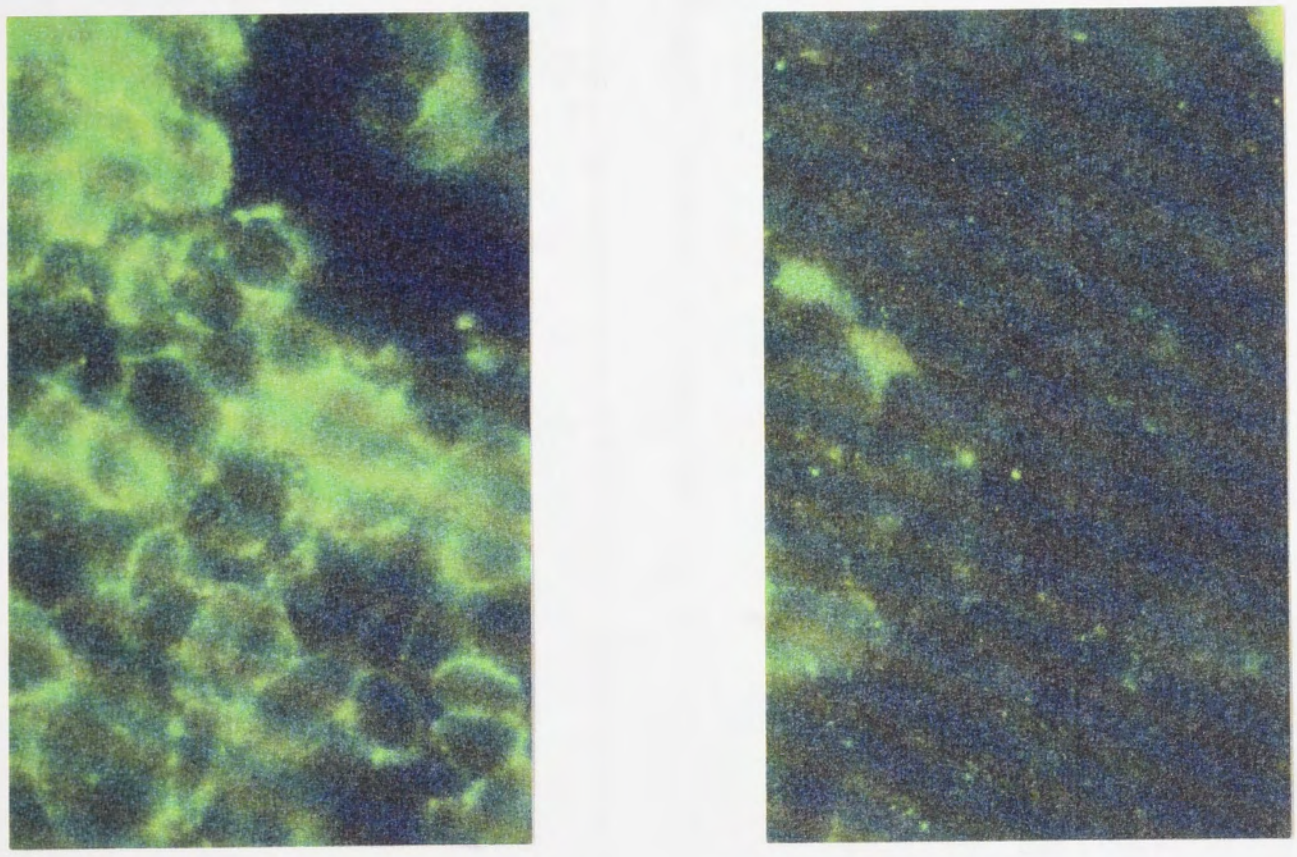
Lanes:

1 - FPV-HA
2 - FPV-HA-IL6
3 - FPV-HA-IFNγ
4 - FPV-M3
5 - λ–*Hin*dIII digests

fragments were not obtained in this study. Insufficient primer elongation time during PCR may account for the inability to amplify for long PCR products.

Expression of HA on the surface of human 143B cells infected with recombinant FPV was shown by immunofluorescence using a rabbit antibody against influenza virus A/PR/8 (Fig. 2.6). Secretion of biologically active IL-6 from monolayers infected with FPV-HA-IL6 was confirmed by bioassay (Table 2.1). Secretion of IFN-γ by cells infected with FPV-HA-IFN-γ was shown by ELISA (Table 2.1). The biological activity of secreted IFN-γ was confirmed by bioassay using WEHI-279 cells (Reynolds *et al.*, 1987; data not included).

Table 2.1 shows daily production of IL-6 and IFN-γ from 143B cells infected with either recombinant FPV or VV. Expression of these factors was more protracted in cultures infected with recombinant FPV than VV. Microscopic examination at day 3 after inoculation revealed that the VV-infected cells had undergone cytopathy, while FPV-infected cells, which are abortively infected, were still viable.



A B

FIGURE 2.6. ***Immunofluorescence of FPV-infected 143B cells***

143B cell monolayers were infected with FPV-HA (A) or FPV-M3 (B) at a multiplicity of infection of 1 for 24 h and were stained with rabbit anti-HA (influenza virus A/PR/8) antisera. Antibodies binding HA were detected using an anti-rabbit immunoglobulin-flourescein conjugate. The monolayers were then mounted on microscope glass slides and examined by ultraviolet microscopy.

[magnification x400]

TABLE 2.1. ***Daily*** **in vitro** ***production of cytokines***[a]

24-h periods	Units of cytokine/ml (mean ± SEM)[b] supernatant from cells infected with:			
	FPV-HA-IL6[c]	VV-HA-IL6[c]	FPV-HA-IFNγ[d]	VV-HA-IFNγ[d]
24	65 301 ± 3 853	31 512 ± 216	650 ± 27	1 175 ± 59
48	21 541 ± 528	9 360 ± 421	370 ± 8	126 ± 5
72	9 057 ± 418	348 ± 13	117 ± 6	< 5[e]
144	4 374 ± 267	< 100[e]	68 ± 2	< 5[e]

[a]Supernatants from 143B monolayers infected at a moi of 1 were passed through 0.1 μm filters to remove virus particles (see Section 2.2.9).

[b]Data shown are the means ± SEM of triplicate supernatant samples. These results were reproduced with another 3 samples.

[c]IL-6 was assayed using the IL-6-dependent cell line, B9 (Aarden *et al.*, 1987). IL-6 was detected at very low background levels from 143B cells infected with control viruses, FPV-HA or VV-HA (Coupar *et al.*, 1988).

[d]IFN-γ activity was assayed by ELISA (Carpenter *et al.*, 1994). No IFN-γ was detected in supernatants overlying cells infected with control viruses.

[e]Limit of detection of the cytokine assay.

2.4 DISCUSSION

Different techniques for construction of recombinant VV are now well-established and widely used. Recombinant viruses are selected using markers, such as the HSV TK (Mackett *et al.*, 1982b; Panicali and Paoletti, 1982), neomycin-resistance (Franke *et al.*, 1985), *Ecogpt* (Boyle and Coupar, 1988b; Falkner and Moss, 1988) or *lac*Z (Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986) gene. These techniques have now been extended to the construction of other recombinant poxviruses that may be useful as vaccine vectors in mammals, namely avipoxviruses (Taylor and Paoletti, 1988; Cox *et al.*, 1993b). In this chapter, the construction of recombinant avipoxviruses, FPV, for delivery of HA and murine cytokines was described.

The FPV genome has not been extensively studied and there are only a few non-essential regions identified for insertion of heterologous genes. The isolation of the TK gene of FPV (Boyle *et al.*, 1987; Binns *et al.*, 1988) and the demonstration that this gene is non-essential for virus replication (Boyle and Coupar, 1988a) has allowed the insertion of foreign genes into this site. In this study, heterologous DNA was inserted in the FPV genome downstream of the TK gene. The presence of repeated sequences, such as P.E/L promoters driving different genes, could contribute to the instability of recombinant viruses as a result of homologous recombination among identical sequences. In order to minimise the occurrence of these recombination events, the viruses were engineered so that similar sequences were placed in opposite directions of each other (Spehner *et al.*, 1990). In this way, homologous recombination is unlikely, thus ensuring the stability of resultant recombinant viruses.

Although infection of FPV in mammalian cells is abortive (Taylor and Paoletti, 1988; Somogyi *et al.*, 1993), genes under the transcriptional control of early virus promoters are expressed (Taylor *et al.*, 1988b; Cox *et al.*, 1993b). Thus, genes for both HA and murine cytokines IL-6 or IFN-γ proximal to the P.E/L FPV promoter were expressed early after infection of a human cell line. Two factors important for optimal expression of heterologous genes in the recombinant viruses were alignment of initiation codons and inclusion of transcriptional termination signals. Kumar and Boyle (1990)

have shown that transcription initiates at the ATG sequences of the P.E/L promoter. Hence, to allow in-frame translation of the inserted gene, the ATG initiation codons of the heterologous gene and the P.E/L promoter were aligned. The utility of the P.E/L promoter would be enhanced if the ATG sequences of the promoter are mutated, thus abolishing the need for initiation codon alignment. However, this could not be achieved as the ATG sequences of the P.E/L promoter are necessary for optimal promoter activity (Boyle, 1992). Expression of both cytokine and HA genes, positioned in opposite directions to each other, was also optimised by inclusion of two overlapping transcriptional termination signals for early expression, i.e. TTTTTTTT (Rohrmann and Moss, 1985; Yuen and Moss, 1987), at the end of each gene. These termination signals reduced the possible modulation of gene expression through the transcription of anti-sense mRNA.

While VV caused destruction of the infected cells, little or no cytopathic effect due to FPV infection was observed. The abortive nature of FPV infection in mammalian cells may be important for the prolonged expression of foreign genes. In this regard, recombinant FPV secreted the encoded cytokines for longer periods than the corresponding recombinant VV. Furthermore, the majority of FPV proteins are not expressed in abortive infections. Therefore, antigenic competition between viral and heterologous proteins is reduced, and the immune response may be directed towards the encoded antigens. The lack of immune responses directed against FPV antigens may also allow persistence of the virus resulting in more prolonged expression of the vaccine antigens in recipient animals. Given that long-lived immune cells may be maintained by persistence of antigen (Gray and Skarvall, 1988; Gray and Matzinger, 1991; Oehen *et al.*, 1992), the prolonged expression of foreign antigens due to persistence of virus may account for the protracted immune responses reported after immunisation with recombinant avipoxviruses (Taylor *et al.*, 1991 and 1992; Cadoz *et al.*, 1992; Cox *et al.*, 1993b; Tartaglia *et al.*, 1993). Furthermore, multiple immunisations are likely to be more effective in situations where there is a reduced primary immune response to the vector itself. Indeed, prior exposure to the vector did not inhibit boosting of antibodies against

rabies virus glycoprotein by recombinant CPV in humans (Cadoz *et al.*, 1992). Moreover, cytokine expression by recombinant VV has markedly altered immune responses generated against the co-expressed heterologous antigen (Ramshaw *et al.*, 1992). However, the adjuvant effect of VV-encoded factors was short-lived (Ramsay *et al.*, 1994a). Taken together, the nature of the abortive infection of FPV and the likelihood of protracted expression of encoded genes point to the potential of recombinant FPV as safe alternative vectors for the induction of protracted immune responses, and prolonged effects of FPV-encoded cytokines on these responses. These possibilities will be examined in Chapter 3.

CHAPTER 3

Selective induction of immune responses by recombinant fowlpox viruses expressing cytokine genes

3.1 INTRODUCTION

FPV, the type species of *Avipoxvirus*, causes abortive infections in mammalian cells (Taylor and Paoletti, 1988; Somogyi *et al.*, 1993). Although unable to replicate in mammals, avipoxviruses can express appropriately engineered foreign genes under the transcriptional control of early virus promoters (Taylor *et al.*, 1988b; Cox *et al.*, 1993b). FPV has recently been developed as a potential vector for the delivery of antigens to mammalian hosts (Taylor and Paoletti, 1988). FPV and another avipoxvirus, CPV (Taylor *et al.*, 1991; Tartaglia *et al.*, 1993), have been used for vaccination of mice (Cox *et al.*, 1993b), cats (Tartaglia *et al.*, 1993) and dogs (Taylor *et al.*, 1991 and 1992). Recently, successful immunisation of humans with recombinant CPV vectors has also been reported (Cadoz *et al.*, 1992).

Effective vaccination lies in the development and maintenance of long-term protective immunity. For many diseases, protection is determined by the type of immune response induced. Whether the immune response is driven towards antibody or cell-mediated responsiveness is critically influenced by the profile of cytokines produced by Th lymphocytes and other immune cells. For example, in helminth (Pearce *et al.*, 1989; Sher and Coffman, 1992; Urban *et al.*, 1992) or toxoplasma infestation (Sher and Coffman, 1992; Sher *et al.*, 1992), or infection with HIV (Maggi *et al.*, 1987; Clerici *et al.*, 1991 and 1993a; Sher *et al.*, 1992; Clerici and Shearer, 1993) or *Listeria* (Magee and Wing, 1988; Bancroft *et al.*, 1989; Tripp *et al.*, 1993), there are strong correlations between Th phenotype dominance and disease susceptibility as outlined in Chapter 1.

In order to modify the immune responses elicited by vaccine antigens, recombinant VV have been engineered to express cytokine genes (Ramshaw *et al.*, 1992; Ramsay and Kohonen-Corish, 1993; Ramsay *et al.*, 1994a and 1994b). Results from studies with these recombinants suggested that the encoded cytokines can either alter the pathogenicity of the vector virus (Karupiah *et al.*, 1990b; Ramshaw *et al.*, 1992) or selectively augment immune responses generated against co-expressed heterologous antigen (Ramsay and Kohonen-Corish, 1993; Ramsay *et al.*, 1994a and 1994b).

In this chapter, the safety of avipoxvirus vectors encoding cytokine genes and their ability to selectively induce immune responses were studied. In particular, the ability of encoded IL-6 and IFN-γ to stimulate antibody and cell-mediated immune responses to co-expressed vaccine antigen was examined.

3.2 MATERIALS AND METHODS

3.2.1 Mice

CBA/H and severe-combined immunodeficient (SCID) mice were obtained from the Animal Breeding Establishment of the John Curtin School of Medical Research. Mice were raised under SPF conditions and were used at 6-8 weeks of age unless stated otherwise.

3.2.2 Culture medium

As described in Section 2.2.1.

3.2.3 Cells

Murine L929 cells, a continuous fibroblast line from the C3H mouse (Sanford *et al.*, 1948), were grown in F15 medium. The human 143B cell line is described in Section 2.2.2.

3.2.4 Viruses

The construction of recombinant FPV was illustrated in Chapter 2. Recombinant FPV and VV were prepared as described in Section 2.2.4. An L929 cell-adapted strain of VV, VV-WR (Wokatsch, 1972), was grown in 143B cells.

Influenza virus strain A/PR/8 was grown and purified according to standard techniques (Jones and Ada, 1986). Briefly, embryonated eggs, 8-10 days old, were infected at 5 hemagglutining units (HAU) of influenza virus/egg for 3 days in a humidified incubator (37°C) with gentle rocking. Allantoic fluid was harvested and titres of virus stocks were determined by hemagglutination. Virus was purified by elution with chicken red blood cells and followed by centrifugation on a sucrose [10-40% (w/v)] gradient before freezing at -75°C.

Lethal challenge with influenza virus was given by i.n. inoculation of 100-fold the median lethal dose (LD_{50}) of mouse-adapted virus {in cold PBS supplemented with FCS [5% (v/v)]; PBS-FCS} into the lungs of mice anaesthetised i.p. with avertin [2% (v/v)

2-methyl-2-butanol, 2% (w/v) 2,2,2-tribromoethanol; 10 µl/g of mouse]. Intravenous inocula of influenza virus comprised 1 x 10^3 HAU in 200 µl of cold PBS-FCS.

3.2.5 Isolation of spleen and lung cells

Lymphoid cells were prepared from spleens as described elsewhere (Karupiah *et al.*, 1990b). Single cell suspensions from pooled spleens were obtained by gently pressing minced spleens through stainless steel mesh with syringe plungers. These cells were washed once in F15 medium, erythrocytes were removed by water lysis and the cells were washed twice. After the final wash, cells were resuspended at the required concentration in F15 medium.

Lung lymphoid cells were prepared as previously outlined (Ramsay and Kohonen-Corish, 1993). Lungs were minced gently into a paste and suspended in F15 medium. Collagenase A (EC 3.4.24.3; BM), dispase II (EC 3.4.24.4; BM) and DNase type II (EC 3.1.22.1; CALBIOCHEM Corporation, CA, USA) were added at concentrations of 2 mg/ml, 1.2 U/ml, and 5 U/ml, respectively, in a final volume of 2 ml/pair of lungs. After incubation at 37°C for 90 min, the lysates were passed gently through an 18-G needle attached to a 20-ml syringe. The resulting suspension was filtered through sterile gauze and washed once with HANKS buffer. Erythrocytes were removed by water lysis and cells were filtered through mesh nybolt nylon filters (pore size, 60 µm; Ure Pacific Pty. Ltd., Vic., Australia). After the final wash, cells were suspended at an appropriate concentration in F15 medium.

3.2.6 CTL assays

Cytotoxic activity was measured in ^{51}Cr release assays (Karupiah *et al.*, 1990b). Target cells were labelled with ^{51}Cr (10 µCi/10^6 cells) at 37°C in a humidified atmosphere containing CO_2 [5% (v/v)] in air. After incubation for 90 min, cells were washed three times in F15 medium without FCS and resuspended at 2 x 10^5 cells/ml of F15 medium for use as targets. For virus-infected target cells, L929 cells were infected with wild-type FPV-M3, VV-WR or recombinant FPV at a moi of 10, or with 5 x 10^{-3} HAU of influenza virus/cell during ^{51}Cr labelling.

CTL assays were performed in triplicate in 96-well U-bottom microtiter plates (Linbro, Virginia, USA) for 6 h at 37°C in a humidified atmosphere of CO_2 [5% (v/v)] in air. ^{51}Cr released from targets was measured in a Packard Auto-Gamma counter and specific lysis was calculated using the following formula:

$$\% \text{ specific lysis} = \frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100\%$$

For secondary CTL assays, 10^8 splenocytes or lung lymphoid cells were isolated. One-fifth of these cells were used as stimulator cells and were infected with 5 x 10^3 HAU of influenza virus, or at a moi of 10 for VV or FPV, for 90 min at 37°C in a volume of 0.5 ml of F15 medium. Infected cells were washed six times in F15 medium before exposure to γ-radiation (2000 rads; ^{60}Co source) and two further washes, prior to use as stimulator cells. The remaining four-fifths of the cells were then cultured with the stimulator cells in a volume of 50 ml MLC for 5 days at 37°C. At the end of this period, cells were harvested and used as effectors in CTL assays.

3.2.7 Antibody assays

Specific anti-HA antibody titres in sera were determined by ELISA (Andrew *et al.*, 1989). ELISA plates (Titertek, Flow Laboratories, The Netherlands) were coated with sucrose-density gradient purified influenza virus (5 HAU/ml) in carbonate buffer (Section 2.2.9) at 4°C overnight. After coating, plates were blocked with skim milk [5% (w/v) in PBS] for 1 h at room temperature. Two-fold serially diluted samples were added to the wells and incubated at 37°C for 90 min. Bound antibody was detected with biotinylated anti-murine Ig antibody followed by incubation with streptavidin-conjugated alkaline phosphatase at 37°C for 1 h in PBS-Tween-20 [0.2% (v/v); Sigma]. The assay was then developed and analysed as described in Section 2.2.9, and results were expressed as the reciprocal of the endpoint dilution of sera. The endpoint was defined as the highest dilution that gave optical density readings above background levels in preimmune sera + (3 SD of background levels).

Antibody-secreting cells (ASC) among lung and spleen lymphoid cell isolates were enumerated using an ELISPOT assay described elsewhere (Sedgwick and Holt, 1986; Ramsay *et al.*, 1994a). Wells of ELISPOT plates (Millititer HA 96-well filtration plate; Millipore Corporation, MA, USA) were coated with sucrose-density gradient purified influenza virus (5 HAU/ml) in carbonate buffer. After blocking with skim milk, as described above, each well was filled with cells in F15 medium and plates were incubated at 37°C for 6 h in a humidified atmosphere of CO_2 [5% (v/v)] in air. After washing cells out of the wells, ELISPOTS were next detected by application of anti-murine Ig antibodies as described above for ELISA assays. The numbers of ASC were enumerated after reaction with the substrate BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium at concentrations of 150 and 300 µg/ml, respectively; Sigma) in carbonate buffer.

3.2.8 Isolation and PCR-detection of viral DNA in organs of mice

Total DNA from organs or tissues of mice was obtained using a modified proteinase K digestion method (Strauss, 1994). Approximately 100 mg of frozen tissue was homogenised using a mortar in an 1.5-ml Eppendorf tube and digested at 58°C using freshly prepared proteinase K (10 mg/ml; Boehringer Mannheim) in 500 µl of digestion buffer {Tris (100 mM; pH 7.5), EDTA (5 mM), NaCl (200 mM) and SDS [0.2% (w/v)]}. After incubation for 12-18 h, the lysate was extracted with an equal volume of Tris (pH 8.0)-buffered phenol-chloroform (50%:50%, v/v) followed by chloroform. DNA was precipitated in ammonium acetate (2 M) and two volumes of isopropanol, and washed with ethanol [70% (v/v)]. After drying, the pellet was dissolved in 100 µl H_2O.

For PCR analysis, 2-µl aliquots of DNA were used. Detection of recombinant FPV-HA was performed by PCR using primers P100 ($^{5'}$GGAGATTTCATCGACTATGAGGAGC$^{3'}$) and P200 ($^{5'}$CGTGTTACACTCATGCATTGATGCG$^{3'}$; see Appendix III) which are homologous to nucleotides 328-352 and 858-882 of the influenza virus HA ORF, respectively. PCR reactions were performed in PCR buffer (Section 2.2.5) using a capillary FTS-Thermal

Sequencer (Corbett Research). The PCR conditions were as follows — 95°C, 5 min (1 cycle): 95°C, 5 sec; 54°C, 2 sec; 72°C, 1 min (45 cycles): 72°C, 5 min (1 cycle). PCR products were analysed by agarose gel electrophoresis.

Southern blotting and probing of DNA fragments were performed under high stringency (Section 2.2.5).

3.2.9 Histology

Organs were fixed in 10% (v/v) neutral buffered formalin before embedding in paraffin. Sections of tissues (4 μm thick) were then stained with haematoxylin and eosin, and mounted in mounting fluid.

3.2.10 Statistical analysis

As described in Section 2.2.11.

3.3 RESULTS

3.3.1 Immunodeficient mice survive infection with recombinant FPV

SCID mice lack functional B and T lymphocytes and are, therefore, highly susceptible to virus infections. These mice were used to test the safety of recombinant FPV. SCID mice were inoculated with 10^8 PFU of FPV-HA i.v. and were monitored for up to 3 months post-immunisation. No mortality occurred in mice given recombinant FPV (Table 3.1). Histological examination of tissues from spleens, ovaries, lungs and livers obtained from infected mice, at 3 weeks post-immunisation, revealed no obvious pathological changes (Fig. 3.1). These results were not affected by the co-expression of cytokine genes: no deaths were recorded following administration of i.v. inocula of either 10^8 PFU of FPV-HA-IL6 or FPV-HA-IFNγ. In contrast, SCID mice died within 5-8 days after infection with 10^7 PFU of VV-HA i.v.

Neonatal CBA/H mice (3-day old) are also highly susceptible to virus infection; however, there was no mortality or morbidity found among neonates immunised i.p. with 10^7 PFU of recombinant FPV (Table 3.1). Furthermore, systemic anti-HA antibodies (i.e. reciprocal end-point titres of 400) were elicited by 3 weeks post-immunisation with 10^7 PFU of FPV-HA i.p. Co-expression of IL-6 or IFN-γ genes had no effect on either the mortality or morbidity, or the levels of antibody responses induced in neonates. In contrast, neonates given 10^7 PFU of VV-HA i.p., all died within 2-4 days from disseminated vaccinal infection.

The use of recombinant FPV in SCID and neonatal mice without mortality demonstrates the safety of these constructs.

3.3.2 Prolonged infection of recombinant FPV *in vivo*

The abortive nature of FPV infection of mammalian cells may allow for the prolonged expression of the heterologous genes. Indeed, it was shown in Chapter 2 that FPV-encoded cytokines were secreted from infected cells for extended periods in culture. However, infectious progeny virus could not be recovered from infected cells, although the presence of viral DNA could be detected by PCR with specific primers. In order to

TABLE 3.1. ***Mortality of mice infected with recombinant viruses***[a]

Mice	Mortality of mice after infection with virus [time of death (days)]:	
	Recombinant FPV[b]	VV-HA
SCID[c]	0/6	6/6 [5, 5, 6, 7, 7, 8]
Neonatal CBA/H[d]	0/6	6/6 [2, 3, 3, 3, 4, 4]

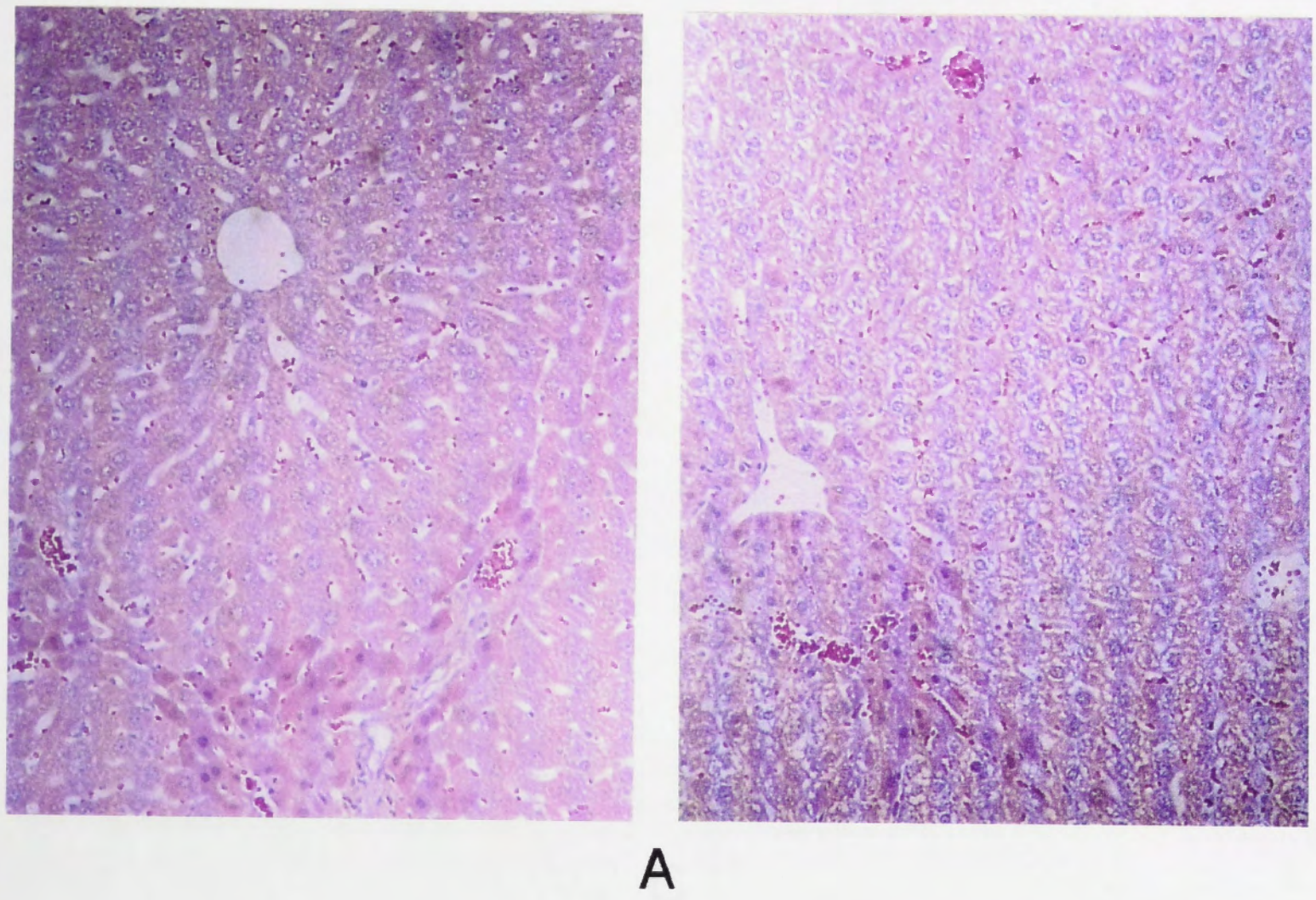
[a]Groups of 6 mice were given the appropriate recombinant viruses and results are representative of 2 experiments.

[b]Each group of mice was given FPV-HA, FPV-HA-IL6 or FPV-HA-IFNγ.

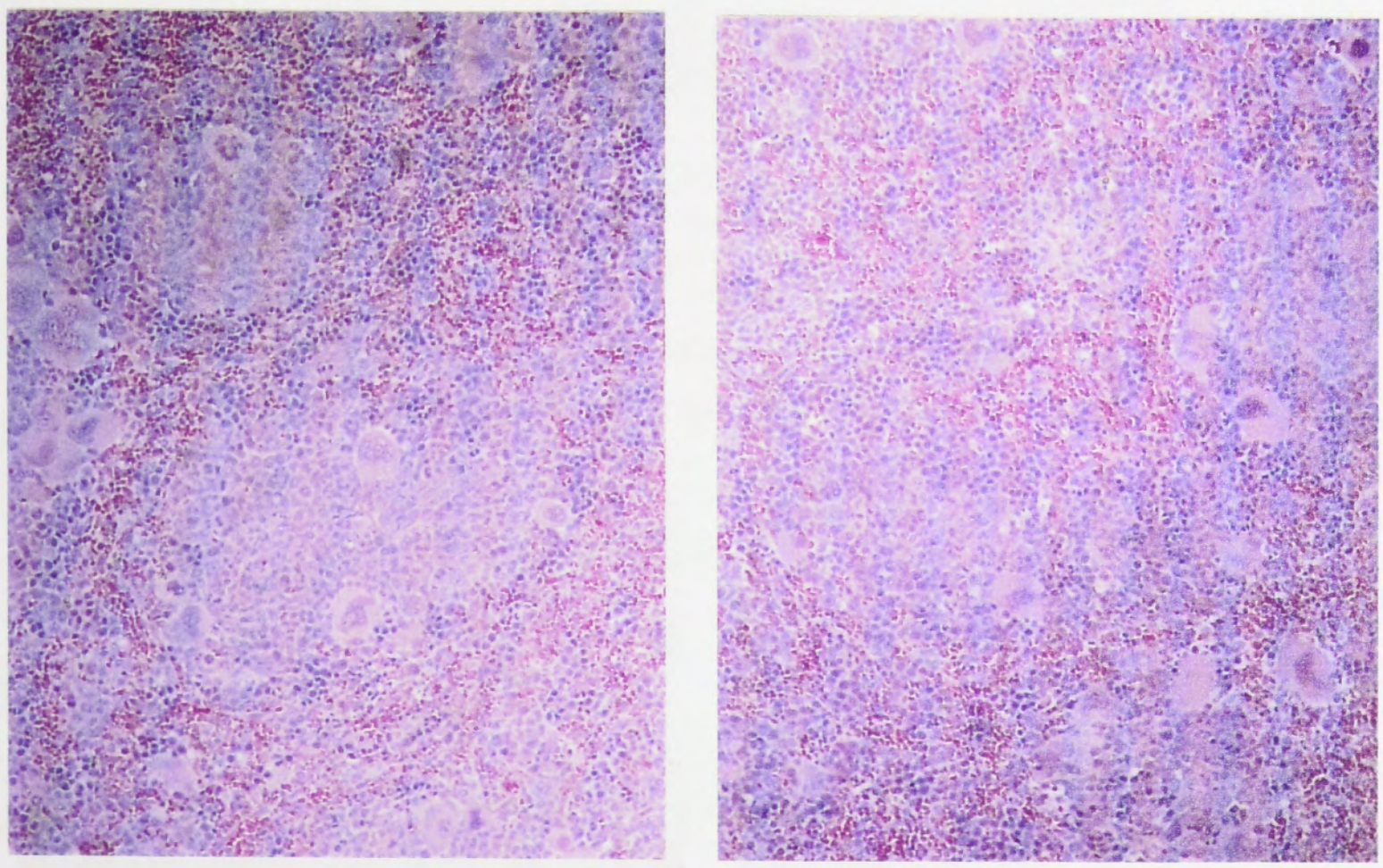
[c]Recombinant FPV and VV-HA were given at 10^8 and 10^7 PFU i.v., respectively, and animals were observed for mortality for 3 months.

[d]Recombinant FPV and VV-HA were given at 10^7 PFU i.p. and animals were observed for mortality for 3 wk.

FIGURE 3.1



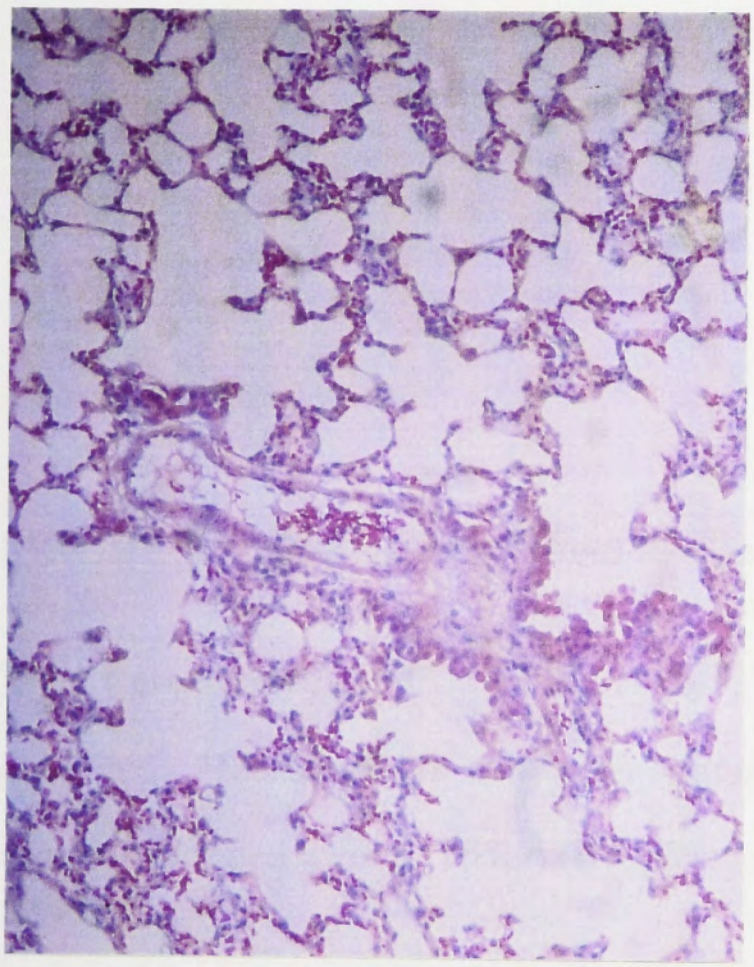
A



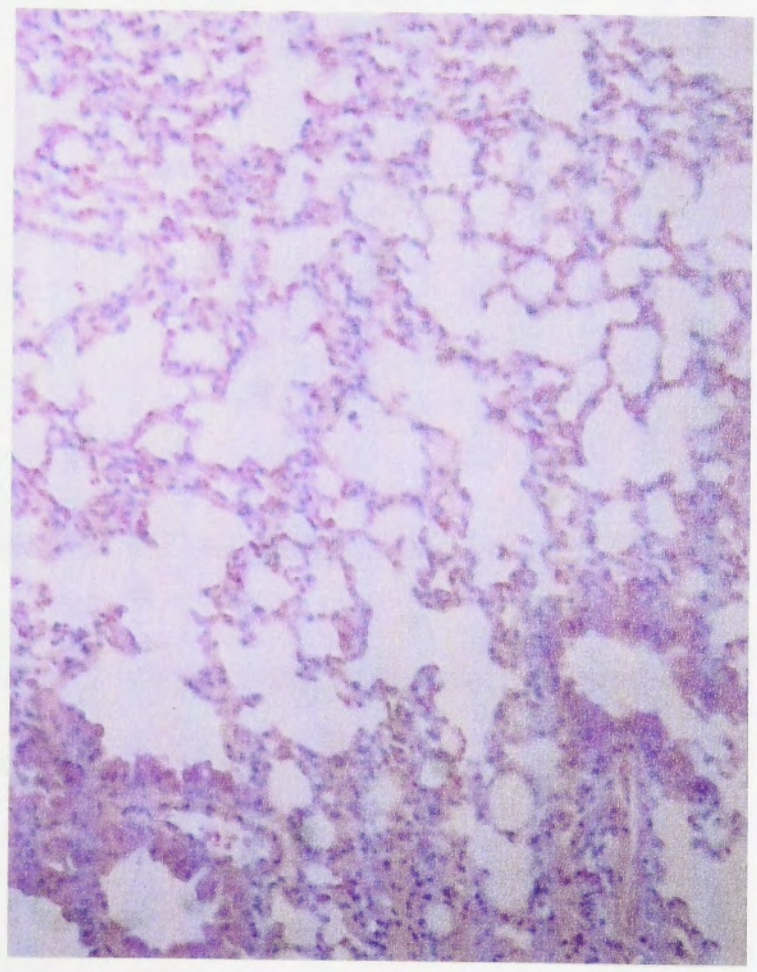
B

FIGURE 3.1

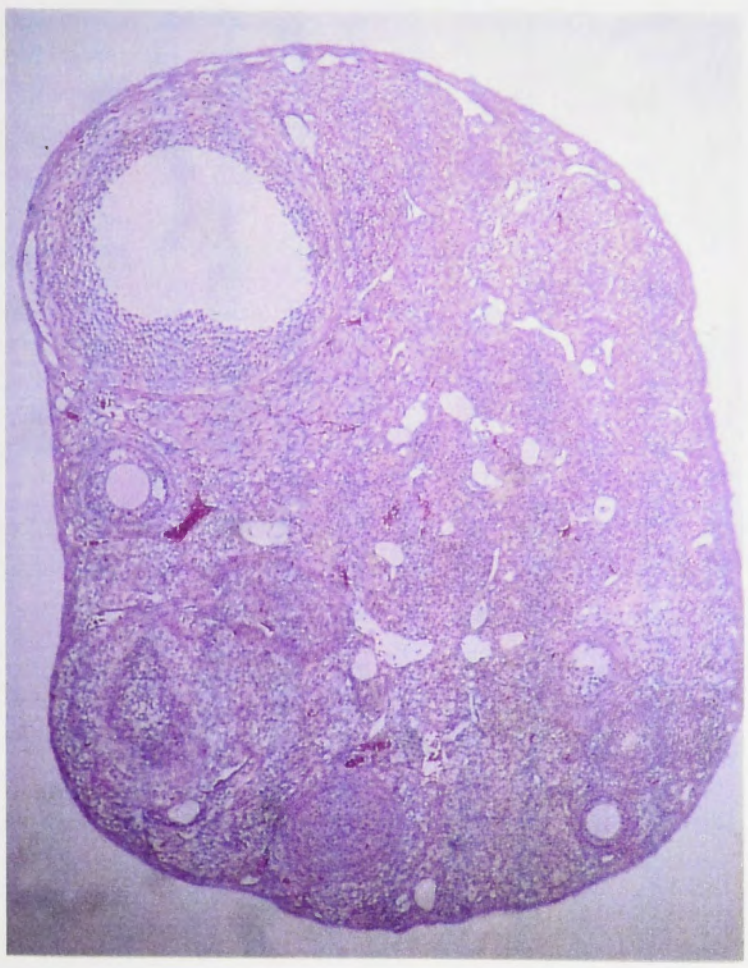
Uninfected

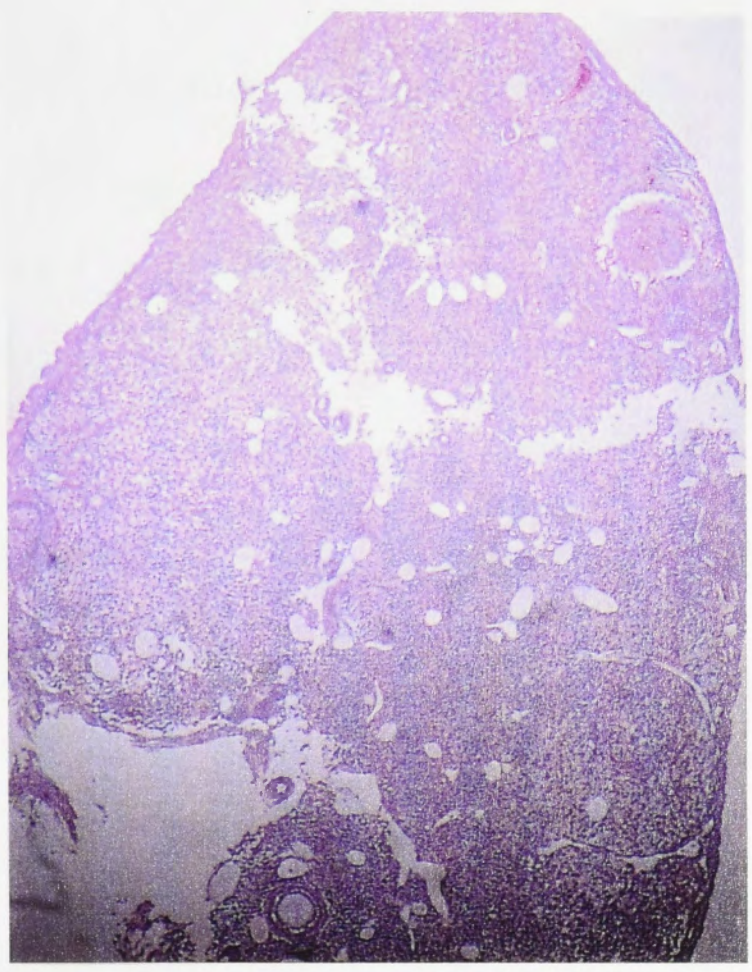


FPV–HA



C





D

FIGURE 3.1. ***Histological changes in organs of SCID mice immunised with FPV-HA***

Mice were given 10^8 PFU of FPV-HA i.v. and at 3 wk post-immunisation, organs were collected. Haematoxylin and eosin-stained histological sections were prepared. The organs examined were (A) liver, (B) spleen, (C) lungs and (D) ovary.

[magnification x100 for (A), (B) and (C), and x40 for (D)]

examine the persistence of recombinant FPV *in vivo*, CBA/H mice were immunised with 10^7 PFU of FPV-HA i.v., and DNA was prepared from livers, spleens, ovaries and lungs. After PCR with primers P100 and P200, a PCR product of ~ 0.5-kb long was found using DNA extracted from lungs, and to a lesser extent, from spleens and liver (Fig. 3.2). As expected, the 0.5-kb PCR fragment was not found after PCR with DNA prepared from unimmunised mice. Thus, recombinant FPV-encoded viral DNA was detected for at least 5 weeks post-immunisation, indicating that FPV can persist *in vivo* for prolonged periods.

3.3.3 Induction of systemic CTL responses

Next, the ability of recombinant FPV to elicit immune responses was studied. No significant primary splenic CTL responses against the FPV vector or co-expressed HA were found at either 1 week (Table 3.2) or 4 weeks (data not shown) after i.v. inoculation with FPV-HA, FPV-HA-IL6 or FPV-HA-IFNγ. Secondary *in vitro* stimulation with influenza virus of splenocytes from immunised mice induced strong CTL responses, however co-expression of cytokines had little effect on the levels of CTL generated. For clarity, only data obtained from mice given control virus, FPV-HA, are shown (Table 3.3). Marked CTL responses against HA and FPV antigens were also found after *in vitro* restimulation of primed splenocytes with the corresponding recombinant FPV used for priming. Again, there were no observable differences in the levels of secondary CTL responses induced in splenocytes taken from mice given recombinant FPV expressing IL-6 or IFN-γ. Interestingly, although secondary CTL could be generated against FPV antigens, these effectors could not lyse VV-infected target cells, showing the lack of CTL cross reactivity between these viruses *in vivo*.

Modest anti-HA and anti-FPV CTL responses were found in mice boosted at week 3 with recombinant FPV. Boosting with VV-HA after priming with FPV-HA also induced anti-HA CTL responses (Table 3.3). Again, no effect due to the co-expression of IL-6 or IFN-γ was observed.

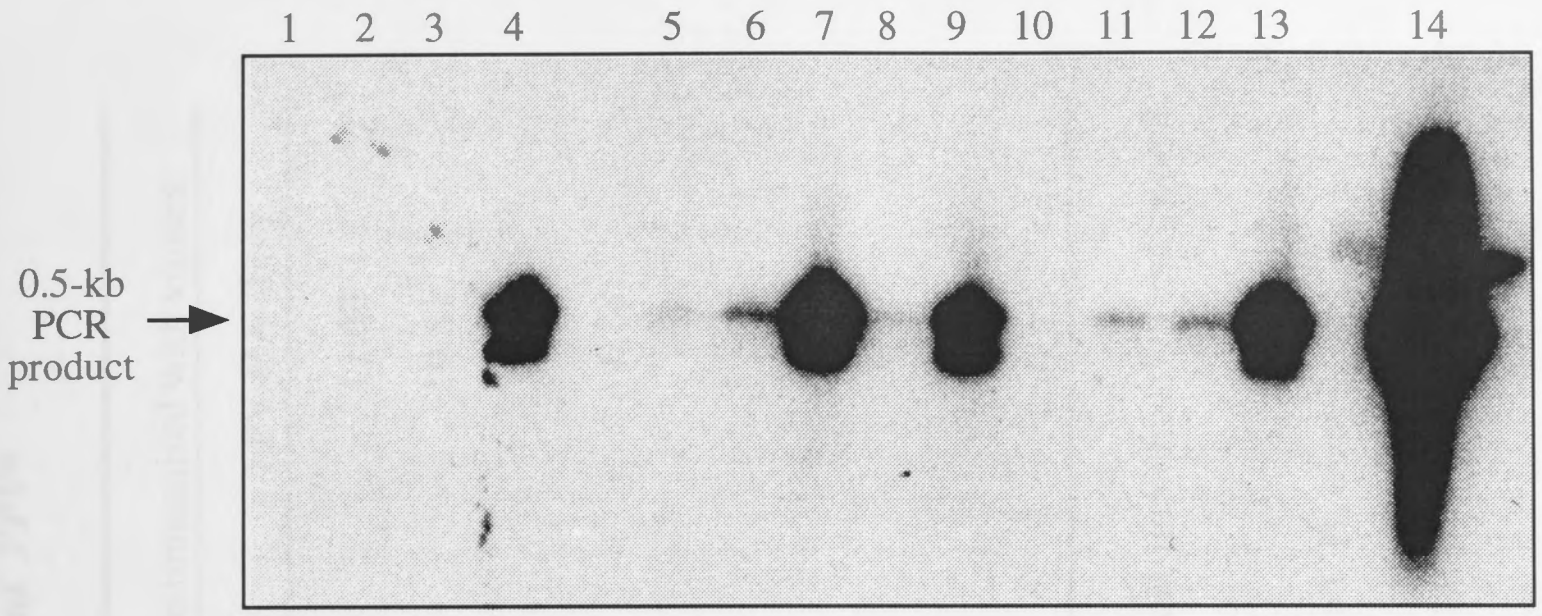


FIGURE 3.2. ***Detection of recombinant FPV DNA by PCR and Southern blotting***

Groups of 4 CBA/H mice were given 10^7 PFU of FPV-HA i.v. and organs were collected at time intervals indicated. DNA from these FPV-infected tissues (100 mg) were prepared according to Section 3.2.8. Tissues from uninfected mice were used as negative controls. After PCR of 10 ng of tissue DNA with primers P100 and P200, PCR products were resolved on a 1.5 % (w/v) agarose gel and followed by Southern blotting of PCR products. Hybridisation was next performed with ^{32}P-labelled HA gene of influenza virus A/PR/8 under high stringency.

Lanes: 1 - uninfected spleen
2 - uninfected liver
3 - uninfected lungs
4 - uninfected lungs + pJZ102 (1 ng)

5 - spleen (wk 1 post-immunisation)
6 - liver (wk 1)
7 - lungs (wk 1)

8 - spleen (wk 3)
9 - lungs (wk 3)
10 - liver (wk 3)

11 - spleen (wk 5)
12 - liver (wk 5)
13 - lungs (wk 5)

14 - pJZ102 (10 ng)

TABLE 3.2. ***Primary splenic CTL responses in mice given recombinant FPV[a]***

CTL[b]	Effector:target ratio	% specific lysis[c] by splenocytes obtained from mice immunised with viruses:		
		FPV-HA	FPV-HA-IL6	FPV-HA-IFNγ
Anti-FPV	60:1	11.13	15.63	11.68
	20:1	7.09	9.46	10.61
	6:1	4.52	9.33	6.18
	3:1	4.58	6.22	6.88
Anti-influenza virus	60:1	1.85	2.44	4.21
	20:1	-3.26	-2.04	-2.00
	6:1	-3.00	0.67	-3.14
	3:1	-1.57	-0.72	-0.27

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant FPV i.v. Spleens were removed at 1 wk post-immunisation and pooled, and antigen-specific CTL assays were performed as described in Section 3.2.6.

[b]L929 target cells were infected with different viruses, as described in Section 3.2.6, for use in antigen-specific CTL assays.

[c]Uninfected L929 cells, used as negative controls, gave low levels of lysis (< 2%). Data shown are means of triplicate wells. SEM for all CTL assays were < 5% and are omitted for clarity. Results are representative of 3 experiments.

TABLE 3.3. ***Secondary systemic CTL responses***[a]

	% specific lysis[b] of L929 cells infected with:			
	Uninfected	FPV-M3	influenza	VV(WR)
In vitro restimulation[c]				
nil	4.9	5.6	15.6	2.7
FPV-HA	14.1	86.1	82.0	18.6
influenza virus	4.6	26.0	73.7	16.0
In vivo boost[d]				
nil	8.1	14.1	5.0	ND[e]
FPV-HA	4.1	22.6	12.0	ND
VV-HA	5.1	8.8	26.3	ND

[a]Groups of 4 CBA/H mice were given 10^7 PFU of FPV-HA i.v. The spleens were collected at times indicated and pooled.

[b]Each point represents the mean of triplicate wells. SEM for all CTL assays were < 5% (omitted for clarity) and results are representative of 3 experiments.

[c]Spleen cells taken from mice at wk 3 post-immunisation were restimulated *in vitro* for 5 days. 33% of the restimulated effector cell cultures were used (see Section 3.2.6).

[d]Mice immunised with 10^7 PFU of recombinant FPV 3 wk earlier were given a booster similar to the priming dose or 10^7 PFU of VV-HA i.v. CTL activity was determined 1 week after boosting. Effector to target ratios were 60:1.

[e]ND, not determined.

3.3.4 Induction of systemic antibody responses

Mice were given 10^7 PFU of recombinant FPV i.v. and primary anti-HA antibody responses were monitored for 12 weeks. At 1 week after priming, responses mounted in mice given FPV-HA-IL6 were approximately 2-fold higher than those measured in animals given FPV-HA, however, little difference in the levels of antibody responses was noted at later times (Table 3.4). Anti-FPV antibody titres were low. Both anti-HA and anti-FPV IgG titres were enhanced after boosting with recombinant FPV, while recall anti-HA antibody titres were markedly elevated upon challenge with wild-type influenza virus. The high anti-HA antibody titres were not sustained and by 9 weeks after boosting had fallen to the maximal levels attained following primary immunisation. Anti-HA antibody levels were higher at this time in mice given FPV-HA-IL6 compared to those receiving FPV-HA.

Following subclass analysis of these responses, it was found that the IL-6-expressing recombinant FPV stimulated heightened IgG1 responses which were maintained for at least 4 weeks (Table 3.5). However, boosting with recombinant FPV or challenge with wild-type influenza virus induced high levels of IgG2a antibody that accounted for the great majority of the IgG response at this stage.

Greatly reduced levels of anti-HA antibody were induced in mice immunised with FPV-HA-IFNγ compared to those found in mice primed with control virus (Table 3.6). Even after boosting, specific antibody levels in mice given FPV encoding IFN-γ were markedly suppressed.

Despite the abortive nature of infection, the results presented here point to the effectiveness of recombinant FPV in priming for protracted immune responses. Moreover, antibody responses, but not CTL responses, were markedly altered by co-expressed cytokines: expression of IL-6 enhanced systemic antibody responses whereas IFN-γ down-regulated these responses.

TABLE 3.4. ***Systemic antigen-specific IgG responses***[a]

Time	Immunisation		Reciprocal of end-point IgG titres (mean ± SEM)[b]	
	Primary	Secondary	anti-HA	anti-FPV
Primary responses:				
wk 1	FPV-HA	nil	17 067 ± 4 267	2 133 ± 533
	FPV-HA-IL6	nil	29 867 ± 11 289	3 733 ± 1 411
wk 4	FPV-HA	nil	42 667 ± 8 533	2 133 ± 533
	FPV-HA-IL6	nil	34 133 ± 8 533	5 333 ± 1 067
wk 12	FPV-HA	nil	10 067 ± 2 133	2 133 ± 533
	FPV-HA-IL6	nil	17 067 ± 4 267	1 067 ± 267
Boosting at 3 wk after immunisation:				
wk 1	FPV-HA	FPV-HA	273 067 ± 68 267	68 267 ± 17 067
	FPV-HA-IL6	FPV-HA-IL6	341 333 ± 68 267	68 267 ± 17 067
wk 9	FPV-HA	FPV-HA	10 667 ± 2 133	17 067 ± 4 267
	FPV-HA-IL6	FPV-HA-IL6	51 200[c]	25 600[c]
Challenge at 3 wk after immunisation:				
wk 1	FPV-HA	influenza	238 933 ± 90 308[d]	5 120 ± 1 067
	FPV-HA-IL6	influenza	307 200 ± 102 400[d]	5 120 ± 2 822
wk 9	FPV-HA	influenza	34 133 ± 8 533[e]	3 200 ± 1 600
	FPV-HA-IL6	influenza	59 733 ± 22 577[e]	2 133 ± 533

TABLE 3.4 (continued)

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant FPV i.v. and antigen-specific antibody responses were determined at time intervals as indicated. At wk 3 post-immunisation, mice were given a booster, similar to the priming dose, or challenged with 10^3 HAU wild-type influenza virus i.v. Recall antibody responses were determined 1 or 9 wk later.

[b]Results shown are the mean ± SEM of reciprocal end-point titres for sera taken from 4 individual mice. Results are representative of 2 experiments.

[c]The titres for 4 sera were the same and hence there were no SEM.

[d]Titre in control groups that were not given recombinant FPV prior to challenge was 25 600.

[e]Titre in control groups that were not given recombinant FPV prior to challenge was 6 667.

TABLE 3.5. ***Systemic anti-HA IgG subclass responses***[a]

Time	Immunisation		Reciprocal of end-point IgG titre (mean ± SEM)[b]	
	Primary	Secondary	IgG1	IgG2a
Primary responses:				
wk 1	FPV-HA	nil	2 133 ± 533	10 667 ± 2 133
	FPV-HA-IL6	nil	17 067 ± 4 627[c]	10 067 ± 2 133
wk 4	FPV-HA	nil	5 333 ± 1 067	25 600[d]
	FPV-HA-IL6	nil	10 667 ± 2 133	25 600[d]
wk 12	FPV-HA	nil	1 867 ± 706	10 667 ± 2 133
	FPV-HA-IL6	nil	2 133 ± 533	10 667 ± 2 133
Boosting at 3 wk after immunisation:				
wk 1	FPV-HA	FPV-HA	34 133 ± 8 533	204 800[d]
	FPV-HA-IL6	FPV-HA-IL6	59 733 ± 22 577	273 067 ± 68 267
wk 9	FPV-HA	FPV-HA	5 333 ± 1 067	10 667 ± 2 133
	FPV-HA-IL6	FPV-HA-IL6	5 333 ±1 067	34 133 ± 8 533
Challenge at 3 wk after immunisation:				
wk 1	FPV-HA	influenza	34 133 ± 8 533[e]	170 667 ± 34 133[f]
	FPV-HA-IL6	influenza	29 867 ± 11 289[e]	204 800[d,f]
wk 9	FPV-HA	influenza	3 733 ± 1 411[g]	25 600[d,h]
	FPV-HA-IL6	influenza	10 667 ± 2 133[g]	42 667 ± 8533[h]

TABLE 3.5 (continued)

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant FPV i.v. and antigen-specific antibody responses were determined at time intervals as indicated. At wk 3 post-immunisation, mice were given a booster, similar to the priming dose, or challenged with 10^3 HAU wild-type influenza virus i.v. Recall antibody responses were determined 1 or 9 wk later.

[b]Each result represents the mean ± SEM of reciprocal end-point titres for sera taken from 4 individual mice. Similar results were obtained in a duplicate experiment.

[c]Significantly different in comparison to mice immunised with FPV-HA ($p < 0.02$ by Student's *t* test).

[d]The titres for 4 sera were the same and hence there were no SEM.

[e]Titre in control groups that were not given recombinant FPV prior to challenge was < 400.

[f]Titre in control groups that were not given recombinant FPV prior to challenge was 25 600.

[g]Titre in control groups that were not given recombinant FPV prior to challenge was < 400.

[h]Titre in control groups that were not given recombinant FPV prior to challenge was 6 667.

TABLE 3.6. ***Systemic anti-HA antibody responses after immunisation with FPV-HA-IFNγ***

Virus	Reciprocal of anti-HA end-point antibody titres (mean ± SEM)[a]					
	Experiment 1[b]			Experiment 2[c]		
	IgG	IgG1	IgG2a	IgG	IgG1	IgG2a
FPV-HA	6 400[d]	600 ± 115	153 600 ± 29 560	38 400 ± 7 390	2 600 ± 1 200	179 200 ± 25 600
FPV-HA-IFNγ	450 ± 126[e]	100[d,e]	2 800 ± 400[e]	8 000 ± 1 600[e]	1 500 ± 200	12 800[d,e]

[a]The mean ± SEM of reciprocal end-point titres for sera taken from 4 individual mice are shown. These results are representative of 3 experiments.

[b]Groups of 4 CBA/H mice were given 2 x 10^7 PFU of recombinant FPV i.v. and anti-HA antibody titres were determined at 1 wk post-immunisation.

[c]Groups of 4 CBA/H mice were given 2 x 10^7 PFU of recombinant FPV i.v. and a booster similar to the priming dose 1 wk later. Anti-HA antibody titres were determined 1 wk after boosting.

[d]The titres for 4 sera were the same and hence there were no SEM.

[e]The suppression of antibody responses in mice given FPV-HA-IFNγ was significant in comparison to mice immunised with control virus ($p < 0.002$ by Student's *t* test).

3.3.5 Induction of mucosal immune responses

The induction of mucosal antibody responses by recombinant FPV was investigated in mice given local inocula of these constructs. For measurements of antibody responses in the lungs, the numbers of lung ASC were enumerated. IgG and IgA ASC numbers have been correlated closely with secreted antibody titres (Berthon *et al.*, 1990).

While no significant antibody responses were detected in spleens of mice given FPV-HA i.n., elevated levels of ASC were found in the lungs. Both IgA and IgG responses were found in the lungs of mice by 1 week after immunisation with FPV-HA. These responses were significantly higher in mice given virus expressing IL-6 (Table 3.7). The IgA responses were 2-6-fold higher in mice given FPV-HA-IL6 than in controls while the increase in IgG responses was less pronounced. As was the case with systemic responses, co-expression of IFN-γ markedly suppressed antibody responses in the lungs.

Mucosal antibody responses were greatly augmented following boosting with FPV-HA-IL6 but, again, were suppressed in mice rechallenged with FPV-HA-IFNγ (Table 3.8). Mucosal anti-HA antibody responses in mice primed with FPV-HA-IL6 were also greatly enhanced following challenge with wild-type influenza virus (Table 3.9). Mice primed with the IFN-γ recombinant FPV produced lower mucosal antibody responses when challenged with influenza virus than those primed with the control virus.

CTL responses induced in the lungs were similar in mice given recombinant FPV (Table 3.10). Thus, expression of IFN-γ selectively suppressed the mucosal antibody response but not the CTL responses.

These results therefore show that recombinant FPV is an effective vector for priming mucosal immune responses when given locally. The effects of FPV-encoded cytokines in modulating immune responses at the mucosae were similar to those observed systemically after i.v. administration of these constructs.

TABLE 3.7. ***Primary mucosal anti-HA antibody responses***[a]

Time	Immunisation	Numbers of anti-HA ASC/10^6 cells (mean ± SD)[b]	
		IgG	IgA
wk 1	FPV-HA	50.4 ± 17.3	21.0 ± 6.0
	FPV-HA-IL6	> 200	122.0 ± 18.0[c]
	FPV-HA-IFNγ	< 10[d]	7.0 ± 2.6
wk 2	FPV-HA	20.0 ± 1.7	13.3 ± 1.2
	FPV-HA-IL6	20.0 ± 1.0	33.3 ± 2.3[c]
	FPV-HA-IFNγ	< 10	< 10
wk 3	FPV-HA	32.3 ± 2.4	10.2 ± 2.9
	FPV-HA-IL6	41.2 ± 3.1[c]	21.4 ± 4.2[c]
	FPV-HA-IFNγ	< 10	< 10

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant FPV i.n. Lungs were collected at times indicated, pooled and anti-HA ASC were enumerated.

[b]Results shown are the mean of ASC ± SD for triplicate samples. The data shown are from a single experiment representative of 3 such experiments.

[c]Significantly different from mice given FPV-HA ($p < 0.01$ by Student's *t* test).

[d]Limit of detection due to limited number of lung cells tested.

TABLE 3.8. ***Recall mucosal anti-HA antibody responses***[a]

	Numbers of anti-HA ASC/10^6 cells at wk post-boosting (mean ± SD)[b]:					
Virus	1		2		3	
	IgG	IgA	IgG	IgA	IgG	IgA
Experiment 1[c]						
FPV-HA	93.3 ± 14.0	40.0 ± 12.0	85.0 ± 30.0	30.0 ± 17.0	ND[e]	ND
FPV-HA-IL6	486.7 ± 11.9[d]	1 320.0 ± 52.9[d]	180.0 ± 47.5[d]	163.7 ± 55.5[d]	ND	ND
FPV-HA-IFNγ	< 20[f]	< 20	< 20	< 20	ND	ND
Experiment 2[g]						
FPV-HA	34.3 ± 6.2	33.3 ± 4.5	ND	ND	9.3 ± 2.2	37.3 ± 3.6
FPV-HA-IL6	262.3 ± 52.1[d]	496.7 ± 70.1[d]	ND	ND	100.3 ± 13.2[d]	142.0 ± 9.1[d]
FPV-HA-IFNγ	ND	ND	ND	ND	ND	ND

TABLE 3.8 (continued)

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant FPV i.n. and an i.n. booster similar to the priming dose at the times indicated. The lungs were collected at times indicated, pooled and anti-HA ASC were determined.

[b]Data shown are mean ± SD of ASC for triplicate wells. In each case, data are from a single experiment representative of 3 such experiments.

[c]Mice were boosted at 1 wk post-immunisation and recall antibody responses in the lungs were determined at 1 and 2 wk after boosting.

[d]Significantly different in comparison to mice given FPV-HA ($p < 0.002$ by Student's *t* test).

[e]ND, not determined.

[f]Limit of detection due to limited numbers of lung cells tested.

[g]Mice were boosted at 3 wk post-immunisation, and recall antibody responses in the lungs were determined at 1 and 3 wk after boosting.

TABLE 3.9. ***Mucosal recall antibody responses after i.n. challenge with influenza virus[a]***

Time post-challenge	Immunisation	Numbers of anti-HA ASC/10^6 cells (mean ± SD)[b]	
		IgG	IgA
wk 1[c]	FPV-HA	59.0 ± 15.6	95.7 ± 47.9
	FPV-HA-IL6	312.2 ± 23.4[d]	569.7 ± 72.6[d]
	FPV-HA-IFNγ	< 20[e]	< 20
wk 3[f]	FPV-HA	11.0 ± 4.1	28.8 ± 9.2
	FPV-HA-IL6	58.3 ± 17.2[d]	97.3 ± 5.5[d]
	FPV-HA-IFNγ	< 20	< 20

[a]Groups of 4 CBA/H mice were immunised with 10^7 PFU of recombinant FPV i.n. and at 3 wk post-immunisation, were challenged with a sublethal dose (10^{-3} HAU) of wild-type influenza virus i.n. Recall antibody responses in lungs pooled from 4 mice were determined at 1 and 3 wk later.

[b]Each figure represents the mean number of ASC ± SD in triplicate wells. The data shown are from a single experiment representative of 3 such experiments.

[c]Anti-HA IgG and IgA ASC numbers in control mice that were not primed with recombinant FPV before challenge were 5.4/10^6 and 9.7/10^6 lung cells, respectively.

[d]The differences when compared with mice given FPV-HA are highly significant ($p < 0.0001$ by Student's *t* test).

[e]Limit of detection due to limited numbers of lung cells tested.

[f]Anti-HA IgG and IgA ASC numbers in control mice that were not primed with recombinant FPV before challenge were 12.3/10^6 and 27.2/10^6 lung cells, respectively.

TABLE 3.10. ***Primary antigen-specific CTL responses in lungs of mice[a]***

Time	Virus	% specific lysis[b] by effectors against target L929 cells infected with:			
		uninfected	FPV-M3	FPV-HA	influenza
wk 1	FPV-HA	11.2	8.3	23.6	31.5
	FPV-HA-IL6	12.7	11.1	22.8	36.2
	FPV-HA-IFNγ	15.0	13.4	26.5	37.7
wk 2	FPV-HA	9.7	8.4	28.4	46.1
	FPV-HA-IL6	14.8	22.9	39.0	49.7
	FPV-HA-IFNγ	6.9	10.5	28.7	42.5

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant FPV i.n. The lungs were collected at times indicated and pooled. CTL responses in these lungs were determined as described in Section 3.2.6.

[b]Data shown are means of triplicate wells and SEM for all CTL assays were < 5%. For clarity, the SEM were not included. Effector to target ratios were 15:1 and results are representative of 3 experiments.

3.3.6 Protection against influenza virus challenge

To test the effectiveness of recombinant FPV vaccination, immunised mice were challenged with a lethal infection of influenza virus. Mice were immunised with wild-type or recombinant FPV i.v. or i.n. and tested for their ability to withstand i.n. challenge with 100 LD_{50} of influenza virus. With the exception of priming with FPV-M3, all other recombinant FPV afforded protection. Mice vaccinated with recombinants FPV-HA or FPV-HA-IL6 were fully protected from lethal virus challenge (Fig. 3.3), however, only 50% of mice immunised with IFN-γ recombinant FPV were protected.

Although immunisation with FPV-HA i.n. and i.v. resulted in protection against mortality following challenge with influenza virus, there was some evidence of morbidity, as measured by transient loss of body mass (Fig. 3.4; Andrew and Coupar, 1988; Ulmer *et al.*, 1993). This phenomenon was also observed in mice primed with FPV-HA-IL6 by the i.n. route, however, mice given this construct i.v. developed no symptoms of morbidity after challenge.

These findings demonstrate the efficacy of recombinant FPV in inducing protective immunity against lethal virus challenge in mice.

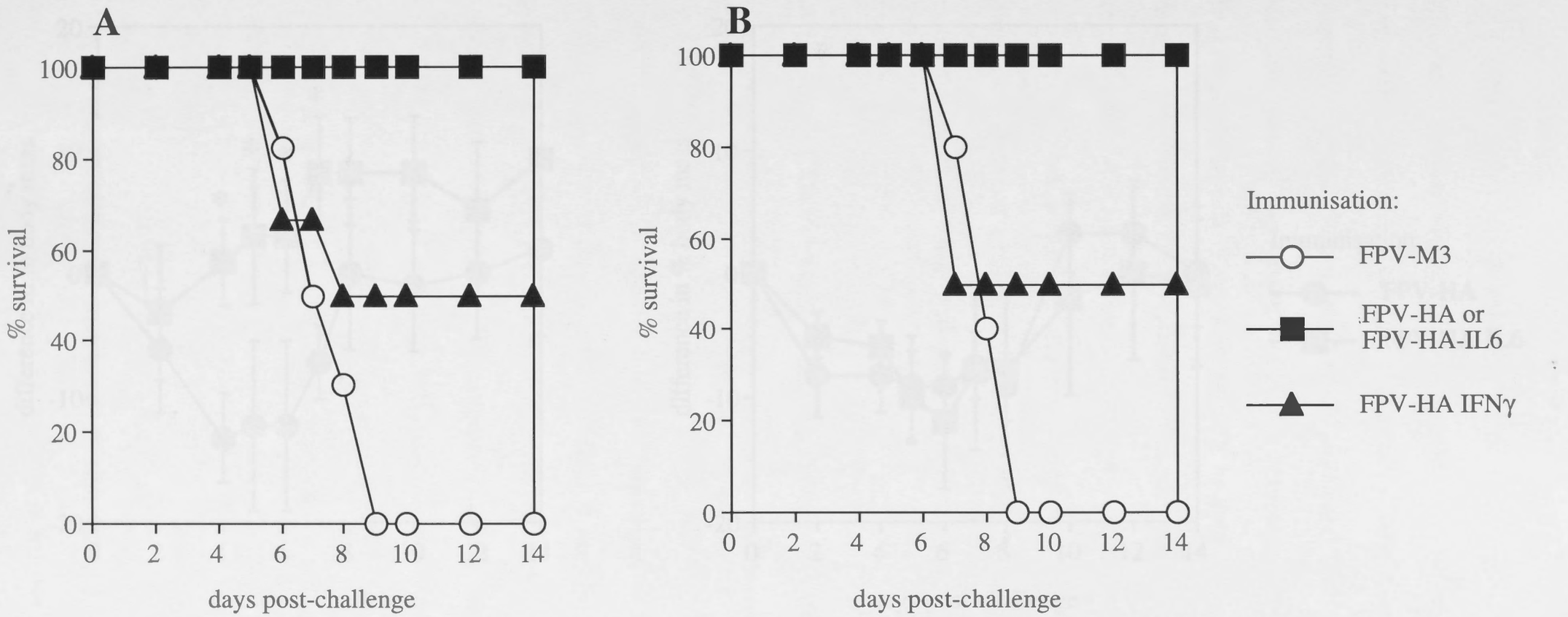


FIGURE 3.3. ***Mortality of mice after challenge with influenza virus***

Groups of 6 CBA/H mice were immunised with 10^7 PFU of recombinant FPV i.v. (A) or i.n. (B). At 3 wk post-immunisation, mice were challenged with 100 LD_{50} of influenza virus i.n. and monitored for mortality.

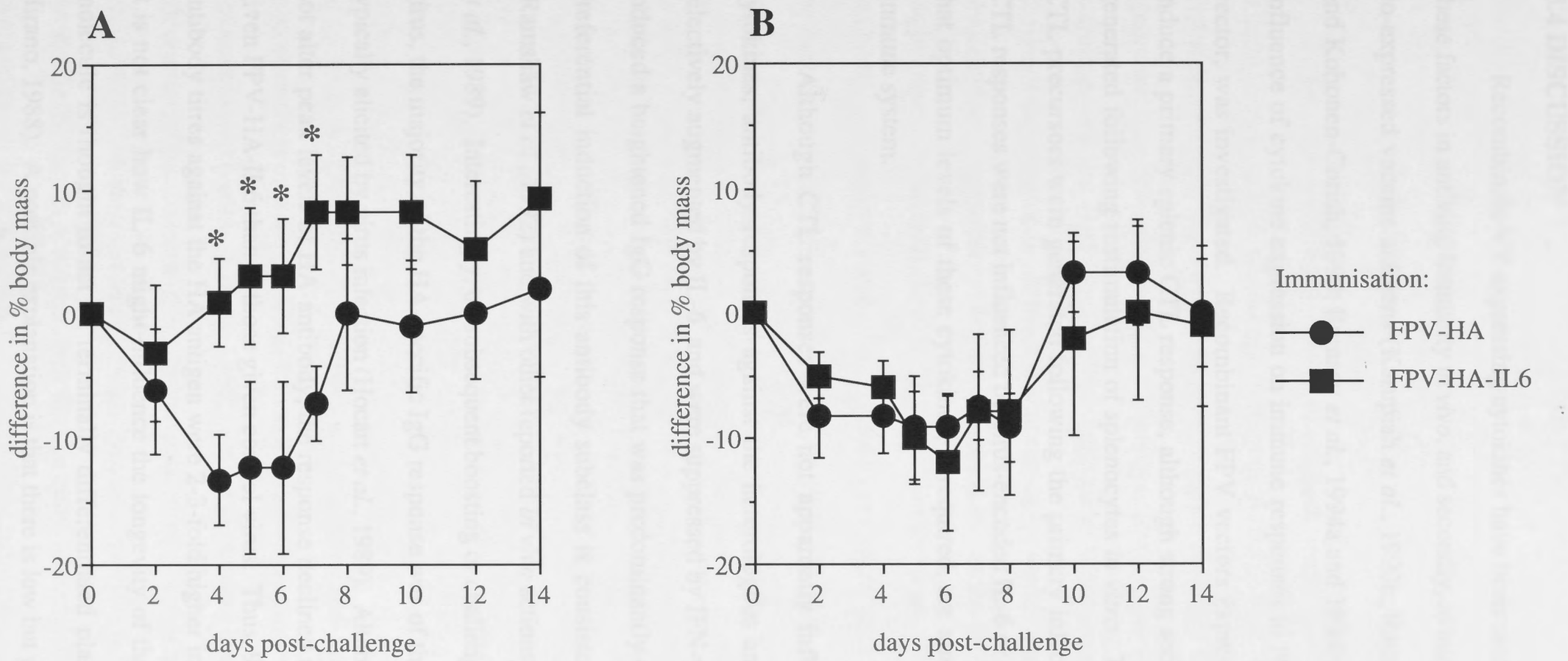


FIGURE 3.4. ***Morbidity of mice after challenge with influenza virus***

Groups of 6 CBA/H mice were immunised with 10^7 PFU of recombinant FPV i.v. (A) or i.n. (B). After 3 wk post-immunisation, mice were challenged with 100 LD_{50} of influenza virus i.n. and monitored for morbidity. This was taken as the mean ± SD of differences in percentage of body mass post-challenge to the original body mass at different times indicated. Results are representative of 2 experiments. *$p < 0.05$, detemined by Student's *t* test in comparison with mice given FPV-HA.

3.4 DISCUSSION

Recombinant VV expressing cytokines have been used firstly, to study the role of these factors in antiviral immunity *in vivo*, and secondly, to modulate immune responses to co-expressed vaccine antigens (Karupiah *et al.*, 1990b; Ramshaw *et al.*, 1992; Ramsay and Kohonen-Corish, 1993; Ramsay *et al.*, 1994a and 1994b). In the present study, the influence of cytokine expression on immune responses to FPV, a non-replicating viral vector, was investigated. Recombinant FPV vectors expressing HA were not able to induce a primary splenic CTL response, although strong secondary responses could be generated following restimulation of splenocytes *in vitro*. This indicates that anti-HA CTL precursors were generated following the primary infection. Levels of secondary CTL responses were not influenced by virus-encoded IL-6 or IFN-γ however, suggesting that optimum levels of these cytokines, if required, are already provided by the host immune system.

Although CTL responses were not apparently influenced by virus-encoded cytokines, antibody responses against the heterologous antigen were markedly and selectively augmented by IL-6, and were suppressed by IFN-γ. The IL-6 encoding virus induced a heightened IgG response that was predominantly of the IgG1 subclass. The preferential induction of this antibody subclass is consistent with previous findings (Ramshaw *et al.*, 1992) and with other reported *in vivo* actions of this cytokine (Suematsu *et al.*, 1989). Interestingly, on subsequent boosting or challenge with wild-type influenza virus, the majority of the HA-specific IgG response was of the IgG2a subclass, which is typically elicited by virus infection (Hocart *et al.*, 1989). Although virus-encoded IL-6 did not alter peak levels of HA-antibody, the response declined much more slowly in mice given FPV-HA-IL6 than in those given control virus. Thus, 9 weeks after the last boost, antibody titres against the HA antigen were 2-3-fold higher in mice given FPV-HA-IL6. It is not clear how IL-6 might influence the longevity of the response, given that this molecule is thought to act on terminally differentiated plasma cells (Kishimoto and Hirano, 1988). A possible explanation is that there is low but prolonged expression of the heterologous genes encoded in recombinant FPV during clearance of the virus. This

notion is supported by the finding that FPV-encoded genes were detected for at least 5 weeks in immunised mice, far longer than is the case for recombinant VV-encoded genes. It should be noted that the adjuvant effect of recombinant VV-encoded IL-6 was relatively short-lived (Ramsay *et al.*, 1994a).

More dramatic enhancement of antibody responses was demonstrated when the recombinant IL-6 FPV was administered i.n. Anti-HA ASC appeared earlier at mucosae and at significantly elevated levels in mice given FPV-HA-IL-6. Antibody responses at mucosae have been shown to be critically dependent on IL-6, as mice with targeted disruption of the IL-6 gene mount negligible such responses following antigenic challenge (Ramsay *et al.*, 1994a). These responses are fully restored, however, following challenge with recombinant VV expressing IL-6. In the experiments reported here, the supplementation of host IL-6 by FPV-encoded cytokine clearly enhances the mucosal antibody response in normal mice. Antibody responses peaked early and were sustained at higher levels in mice boosted with FPV-HA-IL6 rather than control virus. FPV-encoded IL-6 also primed for enhanced local antibody responses to co-expressed HA antigen upon subsequent i.n. challenge with wild-type influenza virus, a finding of potential importance for resistance to infection. Together, these results suggest an important role for IL-6 in the expansion of immune cells during the development of the immune response. Strategies for the delivery of IL-6, such as those described in this thesis, may have major benefits in the development of improved mucosal immunoprophylaxis.

Local IgA antibodies, as well as serum-derived IgG (in the lower tract), are important for protection against infection throughout the respiratory tract, particularly with influenza virus (Ada and Jones, 1986). Findings reported here support an important role for serum IgG antibodies in such protection at mucosae (Couch *et al.*, 1979; Ramphal *et al.*, 1979; McDermott *et al.*, 1982; Prince *et al.*, 1985). Influenza virus challenge locates deep in lungs when delivered to mice under anaesthesia (Yetter *et al.*, 1980). In the present study, mice challenged in this manner were protected against morbidity after priming with IL-6 recombinants given systemically rather than at the mucosae.

Vector-encoded IL-6 markedly augmented specific serum IgG antibody levels when given via systemic routes. The enhanced mucosal IgA responses which did not confer such protection against morbidity in these experiments may, however, be of paramount importance when virus challenge occurs in the URT by means of aerosol (Yetter *et al.*, 1980). Thus, the degree to which serum-derived IgG and local IgA antibodies contribute to prevention of infection may vary according to the site of virus challenge. Nevertheless, both types of antibody responses are clearly elevated by recombinant FPV-encoded IL-6.

Results presented in this study clearly show that expression of virus-encoded IFN-γ suppressed both systemic and mucosal antibody responses. Moreover, IFN-γ recombinant FPV failed to prime mice for recall antibody responses, even after challenge with influenza virus. Previous results show that systemic anti-VV antibody titres were significantly lower in mice infected with IFN-γ-expressing recombinant VV rather than with control VV (Kohonen-Corish *et al.*, 1990). Accordingly, immunisation with FPV-HA-IFNγ afforded poor protection against influenza virus challenge. This result highlights the importance of specific antibody in conferring immunity against homologous influenza virus as reported elsewhere (Askonas *et al.*, 1982). While antibody responses were markedly suppressed in the present study, CTL responses to FPV-HA-IFNγ were as high as those generated by control virus. In general, the ability to selectively generate a strong cell-mediated immune response, as described in this thesis, may have important implications in the development of vaccines against other intracellular pathogens. For example, it has been suggested that the preferential induction of such responses may be of paramount importance for successful vaccination against HIV (Salk *et al.*, 1993).

The safety of recombinant FPV was established in immunodeficient mice and neonates, and expression of cytokine genes did not produce any obvious side effects. Host-restricted FPV abortively infects mammalian cells and is unable to cause disseminated infection in immunodeficient hosts incapable of controlling virus infection. Interestingly, recombinant FPV also primed for specific antibody responses in normal neonatal mice, although the levels of antibody produced were not affected by

vector-encoded IL-6 or IFN-γ. The lack of effect by these cytokines may have been due to the immaturity of lymphoid cells in neonatal mice (Adkins and Hamilton, 1992). Together, these findings have important implications for the development of safe and effective vaccine vectors, including the development of immunisation protocols for infants and immunodeficient individuals.

The nature of the abortive infection may be an important element in promoting immune responses towards the heterologous antigen. Failure to produce FPV proteins would be expected to reduce antigenic competition between viral and heterologous protein, and the immune response would, therefore, be directed towards the encoded antigens driven by early promoters. The poor immune response mounted against FPV may also retard clearance of the virus, allowing more prolonged expression of the vaccine antigens. Furthermore, booster immunisations are likely to be more effective in situations where there is a reduced primary immune response to the vector itself. Indeed, prior exposure to CPV did not inhibit boosting of antibodies against rabies virus glycoprotein by recombinant CPV expressing this antigen in humans (Cadoz *et al.*, 1992).

The finding that there was no immunological cross-reactivity between FPV and VV may have important practical applications. A major problem in utilising VV as a vaccine vector is that a proportion of the population has already been immunised with the virus during the Smallpox Eradication campaign (Fenner *et al.*, 1988). In consequence, these individuals mount lower antibody responses to co-expressed vaccine antigens when given recombinant VV than their non-vaccinated counterparts (Cooney *et al.*, 1991; Etlinger and Altenburger, 1991). The results reported here suggest that this would not be a problem if FPV is used as a vaccine vector. Furthermore, it may be possible to utilise these different vectors encoding the same vaccine antigen to enhance the efficacy of multiple immunisations. Other vectors have been combined successfully in this respect (Li *et al.*, 1993). In the next chapter, the potential of combining immunisation with recombinant FPV and another vector, naked plasmids (Fynan *et al.*, 1993; Montgomery *et al.*, 1993; Ulmer *et al.*, 1993), to increase the efficacy of vaccination will be explored.

In summary, the results presented here demonstrate not only the efficacy and safety of FPV vectors, but also the use of co-expressed cytokines to selectively manipulate the immune response.

CHAPTER 4

Induction of immune responses by

consecutive immunisation with

nucleic acid vaccine and fowlpox virus vectors

4.1 INTRODUCTION

Various replicating viral and bacterial vectors have been developed for delivery of heterologous vaccine antigen (Cavanagh, 1985; Flexner *et al.*, 1988), and the questions of safety and ability to elicit prolonged immunity remain the most important factors governing their use. Replicating viruses that give rise to productive infections in mammalian cells may be harmful when given inadvertently to immunocompromised recipients (Lane *et al.*, 1969; Behbehami, 1983). Moreover, the expression of many vector antigens during infection may lead to the induction of strong immune responses against the vector itself, which may compromise responses against the heterologous antigen. Upon reimmunisation, immune responses against vaccine antigens may be diminished as a result of existing immune responses to the vector (Cooney *et al.*, 1991; Etlinger and Altenburger, 1991). Significant advances have been made towards overcoming these problems, with the development of non-replicating vaccine vectors, namely NAV and avipoxviruses.

Immunisation with NAV has been made feasible with the demonstration that direct gene transfer into mouse muscle *in vivo* resulted in the protracted expression of encoded foreign antigens (Wolff *et al.*, 1990; Wolff *et al.*, 1992; Raz *et al.*, 1993). The vaccine potential of NAV has recently been explored, notably in attempts to develop vaccines against influenza virus (Robinson *et al.*, 1993; Ulmer *et al.*, 1993), HIV (Wang *et al.*, 1993) and malaria (Sedegah *et al.*, 1994). NAV induce low but prolonged specific antibody levels and multiple immunisations are often required to obtain good responses (Fynan *et al.*, 1993; Ulmer *et al.*, 1993). To date, there is no evidence for integration of NAV into the genome of muscle cells (Wolff *et al.*, 1992), or for induction of anti-DNA antibodies that could lead to autoimmunity (Jiao *et al.*, 1992).

In Chapter 3, the development of recombinant FPV encoding cytokine genes as effective vectors for the induction of long-lasting immune responses to heterologous vaccine antigen was described. IL-6 co-expressed by these recombinants augmented antibody responses while IFN-γ inhibited these responses without affecting the generation of cell-mediated immunity. In this chapter, the generation of protective immunity by NAV

immunisation was investigated. This approach was then combined with subsequent immunisation with recombinant FPV in an attempt to enhance immune responses generated by the NAV. The effects of cytokines expressed by recombinant FPV in boosting immune responses were also studied.

4.2 MATERIALS AND METHODS

4.2.1 Mice

As described in Section 3.2.1.

4.2.2 Culture medium

As described in Section 2.2.1.

4.2.3 Cells

As described in Section 3.2.3.

4.2.4 NAV

The plasmid pCMVIA/control (5.5 kb; kindly provided by Dr. H.L. Robinson, University of Massachusetts, MA, USA), constructed from pUC19, contains a CMV IE1 promoter/enhancer with intron A and bovine growth hormone polyadenylation (polyA) signals (Appendix IV). This plasmid has features similar to those of a plasmid vaccine described earlier (Montgomery *et al.*, 1993) and contains a human tissue plasminogen activator (t-PA) signal peptide inserted between the promoter and polyA. Replacement of the t-PA leader with the HA gene of influenza virus A/PR/8 gave rise to pCMVIA/H1. Plasmids were maintained in *E. coli* strain DH5α and purified on CsCl density gradients by standard procedures (Sambrook *et al.*, 1989).

Plasmids, dissolved in sterile saline [0.9% (w/v) NaCl], were injected i.m. into the quadriceps of both hind legs (each quadriceps received one-half of the quantity of NAV specified).

4.2.5 Viruses

As described in Section 3.2.4.

4.2.6 Preparation of splenic and lung lymphoid cells

As described in Section 3.2.5.

4.2.7 Cytotoxicity assays

As described in Section 3.2.6.

4.2.8 Antibody assays

Anti-HA antibody titres were obtained using techniques described in Section 3.2.7. HA-specific antibody was quantified by comparison of the dilution of serum giving half maximal optical density reading with that of a purified mouse Ig standard. The standards used were MOPC 31C (IgG1), UPC 10 (IgG2a), MOPC 141 (IgG2b) and TEPC 183 (IgM; Sigma), and these were applied to ELISA plates coated with the respective sheep anti-mouse Ig subclass antibodies (Southern Biotechnology Associates, Inc., AL, USA). After development with substrate (Section 2.2.9), ELISA results were analysed using the SOFTmax programme version 2.0.1 (Molecular Devices, CA, USA).

4.2.9 Titration of VV

Ovaries from mice were collected and stored in pairs at -70°C. For titration of VV, these tissues were homogenised in 1 ml of cold PBS and an aliquot of 100 µl was incubated with an equal volume of trypsin (1 mg/ml; Sigma) at 37°C for 30 min prior to neutralisation with 800 µl of PBS containing FCS [5% (v/v)]. The digests were then diluted in serials of 10-fold dilution and VV was titrated as plaques on 143B cell monolayers in 6-well plates (Linbro). The monolayers were infected with diluted digests for 1 h before being overlaid with F15 medium. After incubation at 37°C for 2 days in a humidified atmosphere containing CO_2 [5% (v/v)] in air, the monolayers were stained with crystal violet [0.1% (w/v)] in ethanol [20% (v/v)] for 5 min, dried and plaques were counted.

4.2.10 Statistical analysis

As described in Section 2.2.11.

4.3 RESULTS

4.3.1 Induction of primary immune responses after NAV immunisation

The ability of NAV to stimulate specific immune responses was examined by immunisation of mice with an i.m. dose of either 100, 10 or 1 μg of pCMVIA/H1, or 100 μg of the control NAV, pCMVIA/control. Primary systemic anti-HA antibody responses were detected at low levels as early as 1 week post-immunisation with 100 μg pCMVIA/H1 (Table 4.1). Higher antibody titres were detected at week 2 and were sustained for at least 19 weeks post-immunisation. Immunisation with 100 μg induced higher antibody responses than with 10 μg; while at 1 μg, no antibody responses were detected. Only anti-HA antibodies of the IgG2 subclasses were found. Despite these findings of anti-HA antibodies in sera, anti-HA ASC were not found in the spleens after NAV priming (below limit of detection; data not shown).

Primary splenic anti-HA CTL responses could not be detected at either 2 (Table 4.2) or 4 weeks (data not shown) after i.m. inoculation with 100 μg of pCMVIA/H1 or pCMVIA/control. However, upon restimulation with influenza virus *in vitro*, moderate CTL responses against HA were generated among splenocytes taken from mice primed with pCMVIA/H1 (Table 4.3).

These findings demonstrate the ability of NAV to evoke both CTL and antibody responses in mice and confirm the induction of sustained but low levels of systemic antibody as reported by others (Fynan *et al.*, 1993; Ulmer *et al.*, 1993).

4.3.2 Systemic immune responses after NAV immunisation and i.v. boosting with recombinant FPV

In attempts to augment the systemic immune responses generated by NAV, a consecutive immunisation strategy was devised involving priming with NAV i.m. and boosting with recombinant FPV i.v. Four weeks following immunisation with NAV i.m., mice were given a booster inoculum of 10^7 PFU of FPV-HA i.v. High levels of anti-HA antibody were found 1 week after boosting in mice that had been primed with pCMVIA/H1 but not in those given control NAV (Table 4.4). Titres peaked by 3 weeks

TABLE 4.1. ***Primary systemic antibody responses in mice given NAV i.m.***[a]

Time	Subclass	Anti-HA antibody levels (mean ± SD of μg/ml)[b] in mice given: pCMVIA/control	pCMVIA/H1		
		100 μg	100 μg	10 μg	1 μg
wk 1	IgG2a	< 0.05[c]	0.13 ± 0.01	< 0.05	< 0.05
	IgG2b	< 0.05	0.15 ± 0.01	ND[e]	ND
	IgG1	< 0.05	< 0.05	< 0.05	< 0.05
	IgM	< 0.05	< 0.05	< 0.05	< 0.05
wk 2	IgG2a	< 0.05	4.38 ± 1.10[d]	1.18 ± 1.62	< 0.05
	IgG2b	< 0.05	9.26 ± 2.26	ND	ND
	IgG1	< 0.05	< 0.05	< 0.05	< 0.05
	IgM	< 0.05	< 0.05	< 0.05	< 0.05
wk 4	IgG2a	< 0.05	4.75 ± 1.92	2.25 ± 2.06	< 0.05
	IgG1	< 0.05	< 0.05	< 0.05	< 0.05
wk 5	IgG2a	< 0.05	7.26 ± 1.43	5.70 ± 4.65	< 0.05
	IgG1	< 0.05	< 0.05	< 0.05	< 0.05
wk 13	IgG2a	< 0.05	15.2 ± 3.5	9.4 ± 5.1	< 0.05
	IgG1	< 0.05	< 0.05	< 0.05	< 0.05
wk 19	IgG2a	< 0.5	3.4 ± 0.8	2.9 ± 0.5	< 0.5
	IgG1	< 0.05	< 0.05	< 0.05	< 0.05

TABLE 4.1 (continued)

[a]Groups of 5 CBA/H mice were given the indicated quantity of NAV i.m. (each hind leg receiving half the amount) under anaesthesia. At the times indicated, mice were bled and anti-HA antibody responses were measured by ELISA as outlined in Section 4.2.8.

[b]Each result represents the mean ± SD in μg of anti-HA antibodies/ml for each group of mice. Results are representative of 2 experiments.

[c]Limit of detection.

[d]Significantly different from mice given 10 μg of plasmids ($p < 0.007$ by Student's *t* test).

[e]ND, not determined.

TABLE 4.2. ***Primary systemic CTL responses in mice given NAV i.m.***[a]

L929 targets infected with:	Effector:target ratios	% specific lysis[b] of target cells by splenocytes taken from mice infected with:		
		Uninfected	pCMVIA/control	pCMVIA/H1
Influenza virus	60:1	7.93	5.66	8.99
	20:1	3.20	2.44	5.84
	6:1	0.07	7.33	4.18
	2:1	-0.12	0.02	1.54
Uninfected	60:1	6.04	8.92	6.29
	20:1	0.88	6.02	8.08
	6:1	-0.13	-0.13	2.65
	2:1	-0.75	-0.75	0.81

[a]Groups of 4 CBA/H mice were given 100 μg of NAV i.m., and spleens were harvested 1 wk later and pooled. CTL responses were determined as described in Section 4.2.7.

[b]Data shown are mean values from triplicate wells. SEM were < 5% and are omitted for clarity. Data are representative of 3 experiments.

TABLE 4.3. ***Secondary systemic antigen-specific CTL responses after NAV immunisation[a]***

% of restimulated culture	% specific lysis[b] of L929 target cells infected with:			
	uninfected	influenza	FPV-M3	FPV-HA
100	25.85	59.17	27.00	48.87
33	9.47	49.94	22.11	29.73
10	3.39	28.80	15.47	10.74
3	0.85	13.14	7.98	6.53

[a]Groups of 4 CBA/H mice were given 100 μg of pCMVIA/H1 i.m., and 4 wk later, spleens were harvested and pooled. Splenocytes were then restimulated in culture with influenza virus-infected syngeneic stimulator cells for 5 days.

[b]Levels of specific lysis of L929 targets by splenocytes taken from mice immunised with the control NAV and restimulated *in vitro* with influenza virus were 3-10%. Data shown are mean values from triplicate wells. SEM were < 5% and are omitted for clarity. The data shown are from a single experiment representative of 3 such experiments.

TABLE 4.4. ***Recall systemic anti-HA antibody responses after consecutive immunisation with NAV and FPV-HA[a]***

Time post-boosting (wk)	Subclass	Anti-HA antibody (mean ± SD of μg/ml)[b] elicited after boosting with FPV-HA:			
		pCMVIA/control[c]	pCMVIA/H1		
		100 μg	100 μg[d]	10 μg[d]	1 μg[c]
1	IgG2a	16.0 ± 3.5	533.6 ± 151.9	433.5 ± 400.4	19.1 ± 6.6
	IgG1	< 0.05[e]	< 0.05	< 0.05	< 0.05
3	IgG2a	18.3 ± 1.4	1 061.4 ± 347.5[f]	510.8 ± 42.5	22.9 ± 2.0
	IgG1	2.4 ± 0.7	5.9 ± 1.3	3.2 ± 1.0	4.4 ± 2.1
6	IgG2a	8.3 ± 2.9	559.5 ± 217.8	373.9 ± 31.2	17.5 ± 5.4
	IgG1	2.8 ± 1.1	8.1 ± 3.7	5.9 ± 1.3	1.7 ± 0.4
15	IgG2a	13.2 ± 1.4	235.2 ± 122.2	197.0 ± 16.9	9.3 ± 4.6
	IgG1	6.9 ± 1.4	6.6 ± 0.2	5.1 ± 2.4	5.9 ± 1.2

TABLE 4.4 (continued)

[a]Groups of 5 CBA/H mice were given the appropriate NAV i.m. At wk 4 post-immunisation, mice were boosted with 10^7 PFU of FPV-HA i.v. and anti-HA antibody responses were determined at time intervals as indicated.

[b]Each result shown is representative of the mean ± SD in μg of anti-HA antibodies/ml in sera of 5 individual mice.

[c]Titres in control groups that were boosted with control virus not expressing HA, FPV-M3, were < 0.05 μg/ml (limit of detection).

[d]Titres in control groups that were boosted with control virus not expressing HA, FPV-M3, were 2.2-18.2 μg/ml.

[e]Limit of detection.

[f]Significantly different when compared with mice given 10 μg of pCMVIA/H1 ($p < 0.008$ by Student's *t* test).

post-boosting and high levels were maintained for up to 15 weeks. Doses of 100 μg pCMVIA/H1 primed for a greater recall response than 10 μg whilst 1 μg of HA-encoding NAV failed to prime the mice. The antibody subclass elicited was mostly IgG2a and levels of antibodies of the IgG1 subclass were not augmented. Antibody responses in mice primed with pCMVIA/H1 and boosted with recombinant FPV were 30-120-fold higher than in mice vaccinated sequentially with HA-encoding NAV and FPV-M3 wild-type, or control NAV and FPV-HA. Accordingly, augmented numbers of antigen-specific ASC were detected in spleens of mice consecutively immunised with NAV and FPV (Table 4.5).

Again, primary splenic anti-HA CTL responses were not detected in mice given both pCMVIA/H1 (100 μg) and 10^7 PFU of FPV-HA i.v. (data not shown). Data similar to those in Table 4.2 were obtained.

These findings reveal the success of the consecutive immunisation protocol in markedly elevating antibody responses induced by NAV given i.m., however this combined strategy had no effects in enhancing the levels of primary CTL responses generated.

4.3.3 Mucosal immune responses after NAV immunisation and i.n. boosting with recombinant FPV

Previous reports have suggested that NAV given systemically may prime for protective immunity at the mucosae (Fynan *et al.*, 1993; Montgomery *et al.*, 1993; Ulmer *et al.*, 1993). In order to investigate this effect further, mice primed with NAV i.m. 4 weeks earlier were given i.n. inocula of 10^7 PFU of recombinant FPV. Although ASC were not found in the lungs after immunisation with 100 μg of pCMVIA/H1 (below limit of detection; data not shown), both mucosal anti-HA IgG (particularly IgG2a) and IgA antibody responses were markedly enhanced in pCMVIA/H1-immunised mice after boosting with recombinant FPV (Table 4.6). These responses were still present at 3 weeks post-boosting. Immunisation with 10 μg of pCMVIA/H1 failed to prime for subsequent boosting with recombinant FPV i.n. and only data obtained from

TABLE 4.5. ***Recall systemic anti-HA antibody responses after consecutive immunisation with NAV and FPV-HA[a]***

Primary	Secondary	Anti-HA ASC/10^6 cells (mean ± SD)[b]	
		IgG1	IgG2a
100 μg control	FPV-HA	14.0 ± 3.5	105.0 ± 26.0
1 μg pCMVIA/H1	FPV-HA	15.0 ± 5.2	93.0 ± 5.2
10 μg pCMVIA/H1	FPV-HA	13·2 ± 1.7[c]	216.0 ± 32.5[c,d]

[a]Groups of 4 CBA/H mice were given NAV i.m. At 4 wk post-immunisation, mice were boosted with 10^7 PFU of FPV-HA i.v. and anti-HA ASC in the pooled spleens were enumerated 1 wk later.

[b]Data shown are means ± SD of ASC numbers from triplicate wells. The results are representative of 2 experiments.

[c]Anti-HA ASC numbered < $2/10^6$ (limit of detection) cells in mice immunised only with NAV i.m.

[d]The ASC numbers obtained using mice immunised with 10 μg of pCMVIA/H1 were significantly different when compared with those from mice given control NAV ($p < 0.002$ by Student's *t* test).

TABLE 4.6. ***Mucosal anti-HA antibody responses after consecutive immunisation with NAV and recombinant FPV[a]***

Time post-boosting	Immunisation	Boosting	Numbers of anti-HA ASC/10^6 cells (mean ± SD)[b]		
			IgG1[c]	IgG2a[c]	IgA[c]
wk 1	pCMVIA/H1	FPV-HA	< 2	382.2 ± 46.8[d]	195.6 ± 53.9[d]
	pCMVIA/H1	FPV-HA-IL6	93.9 ± 13.3	1 324.4 ± 93.7[e]	768.9 ± 46.8[e]
	pCMVIA/H1	FPV-HA-IFNγ	< 2	850.0 ± 87.2[f]	350.0 ± 30.0[f]
	pCMVIA/control	FPV-HA	< 2	145.5 ± 10.5	60.6 ± 21.0
	pCMVIA/control	FPV-HA-IL6	< 2	359.8 ± 111.5	189.0 ± 40.2
	pCMVIA/control	FPV-HA-IFNγ	< 2	28.6 ± 9.0	15.6 ± 7.8
wk 3	pCMVIA/H1	FPV-HA	< 2	135.0 ± 15.0	282.5 ± 23.3
	pCMVIA/H1	FPV-HA-IL6	25.0 ± 5.0	540.0 ± 63.8[e]	793.8 ± 63.4[e]
	pCMVIA/H1	FPV-HA-IFNγ	< 2	155.0 ± 27.8[g]	327.5 ± 23.3[h]
	pCMVIA/control	FPV-HA	< 2	< 2	< 2
	pCMVIA/control	FPV-HA-IL6	8.3 ± 3.6	63.3 ± 20.8	87.5 ± 18.5
	pCMVIA/control	FPV-HA-IFNγ	< 2	< 2	< 2

TABLE 4.6 (continued)

[a]Groups of 5 CBA/H mice were given 100 μg of NAV i.m. At wk 4 post-immunisation, mice were boosted with 10^7 PFU of recombinant FPV i.n. and anti-HA antibody responses in the pooled lungs were determined.

[b]Data shown represent the mean ± SD of triplicate wells. Similar results were obtained in another 2 experiments.

[c]Numbers of anti-HA ASC in control groups that were boosted with control virus not expressing HA, FPV-M3, were < $2/10^6$ cells (limit of detection).

[d]Significant differences when compared with mice immunised with pCMVIA/control and boosted with FPV-HA ($p < 0.02$ by Student's *t* test).

[e]The differences in ASC numbers were highly significant when compared with groups that were immunised with pCMVIA/H1 and FPV-HA (boost), or pCMVIA/control and FPV-HA-IL6 (boost) ($p < 0.0003$ by Student's *t* test).

[f]The differences in ASC numbers were highly significant when compared with groups that were immunised with pCMVIA/H1 and FPV-HA (boost), or pCMVIA/control and FPV-HA-IFNγ (boost) ($p < 0.0001$ by Student's *t* test).

[g]$p > 0.05$, determined by Student's *t* test in comparison to mice boosted with FPV-HA.

[h]$p < 0.02$, determined by Student's *t* test in comparison to mice boosted with FPV-HA.

immunisation with 100 μg of NAV are shown. The major subclasses of specific anti-HA antibody found in the lungs were IgG2a and IgA.

Interestingly, FPV-HA-IFNγ that primed mice poorly for antibody responses in the lungs (Sections 3.3.4 and 3.3.5; Tables 3.6-3.9), boosted recall responses when given to mice that had been primed with pCMVIA/H1 (Table 4.6). The augmented recall responses found after boosting with recombinant FPV were further elevated when recombinant FPV expressing IL-6 was used. Boosting with FPV-HA-IL6 also induced an IgG1 antibody response.

Priming with FPV-HA i.n. usually resulted in specific local immunity in the lungs (Section 3.3.5). Interestingly, boosting at the mucosae with recombinant FPV after pCMVIA/H1 immunisation also induced systemic antibody responses. Higher anti-HA IgG2a antibodies were found in sera of mice immunised with pCMVIA/H1 i.m. and boosted with an i.n. dose of recombinant FPV (Table 4.7) compared to mice given control NAV and recombinant FPV. In addition, systemic anti-HA IgG1 antibodies were also detected after boosting with i.n. immunisations of recombinant FPV. The IL-6-encoding recombinant FPV, when given i.n., primed for systemic IgG1 antibodies and this finding confirms the induction of IgG1 antibody by vector-encoded IL-6 (Section 3.3.4; Ramshaw *et al.*, 1992). However, immunisation with pCMVIA/H1 before boosting did not further elevate this response. Recall systemic antibody responses diminished at week 3 post-boosting (below limit of detection; data not shown).

Primary anti-HA CTL responses in the lungs were generated after i.n. inoculation with recombinant FPV, as previously shown (Section 3.3.5; Table 3.10), however these responses were not enhanced in mice immunised consecutively with pCMVIA/H1 (100 μg) and i.n. boosting with FPV-HA (Table 4.8).

The data presented in this section point to the ability of systemic immunisation with NAV to prime for specific mucosal antibody responses. The responses were augmented following local boosting with recombinant FPV. Further enhancement of

TABLE 4.7. ***Systemic anti-HA antibody titres after consecutive immunisation with NAV and recombinant FPV i.n.***[a]

Immunisation	Boosting	Anti-HA antibody titers (μg/ml ± SD)[b]	
		IgG1	IgG2a
pCMVIA/H1	FPV-M3	< 0.1[c]	2.1 ± 0.3
pCMVIA/H1	FPV-HA	0.2 ± 0.1	19.5 ± 8.1[d]
pCMVIA/H1	FPV-HA-IL6	5.3 ± 2.4[e]	22.0 ± 9.5[d,f]
pCMVIA/H1	FPV-HA-IFNγ	< 0.1	13.9 ± 9.1[d,f]
pCMVIA/control	FPV-M3	< 0.1	< 0.1
pCMVIA/control	FPV-HA	< 0.1	2.1 ± 1.8
pCMVIA/control	FPV-HA-IL6	3.2 ± 0.2	4.0 ± 1.0
pCMVIA/control	FPV-HA-IFNγ	< 0.1	0.3 ± 0.1

TABLE 4.7 (continued)

[a]Groups of 5 CBA/H mice were given 100 μg of NAV i.m. At wk 4 post-immunisation, mice were boosted with 10^7 PFU of recombinant FPV i.n. and anti-HA antibody levels in serum were determined 1 wk later.

[b]Each point represents the mean ± SD of anti-HA antibodies titres for 5 individual mice. Results are typical of 2 experiments.

[c]Limit of detection.

[d]Significantly different in comparison to groups primed with control NAV and boosted with respective recombinant FPV ($p < 0.01$ by Student's *t* test).

[e]The levels of IgG1 after immunisation with pCMVIA/H1 and FPV-HA-IL6 were not significantly different to the control group given NAV control and FPV-HA-IL6 ($p > 0.05$ by Student's *t* test).

[f]Not significant in comparison to mice boosted with FPV-HA ($p > 0.5$ by Student's *t* test).

TABLE 4.8. ***Antigen-specific CTL responses in lungs of mice immunised with NAV and recombinant FPV i.n.***[a]

Immunisation	Boosting	% specific lysis[b] by effectors against target L929 cells infected with:			
		uninfected	FPV-M3	FPV-HA	influenza
pCMVIA/control	FPV-HA	6.5	5.1	33.6	27.6
	FPV-HA-IL6	2.8	8.2	29.3	30.1
	FPV-HA-IFNγ	5.2	2.1	31.9	28.2
pCMVIA/H1	FPV-HA	9.2	7.7	35.1	30.5
	FPV-HA-IL6	11.5	9.1	27.1	34.5
	FPV-HA-IFNγ	7.0	10.2	29.5	38.1

[a]Groups of 4 CBA/H mice were given 100 μg of NAV i.m. and at 4 wk post-immunisation, were boosted with 10^7 PFU of recombinant FPV i.n. The lungs were collected 1 wk post-boosting and pooled. CTL responses in these lungs were determined.

[b]Data shown are means of triplicate wells and SEM for all CTL assays were < 5%. For clarity, the SEM were not included. Effector to target ratios were 15:1 and results are representative of 2 experiments.

mucosal responses was achieved when IL-6 was encoded by the boosting vector. Again, with this sequential vaccination strategy, pulmonary CTL responses were not augmented.

4.3.4 Protection against influenza virus challenge

The ability of NAV to protect against lethal challenge with influenza virus when used alone or in combination with recombinant FPV was examined. Mice immunised with 100 μg of pCMVIA/H1 alone or in combination with 10^7 PFU of recombinant FPV were tested for their ability to withstand i.n. challenge with 100 LD_{50} of influenza virus. All mice given pCMVIA/H1 alone were protected from mortality upon lethal challenge with influenza virus unlike those given control NAV (Fig. 4.1). Although mice given pCMVIA/H1 i.m., or control NAV followed with FPV-HA (either i.n. or i.v.), were protected from mortality, these mice developed transient symptoms of influenza as determined by loss of body mass (Fig. 4.2). In contrast, no significant loss of body mass or other obvious signs of morbidity were observed in mice immunised with pCMVIA/H1 and boosted with FPV-HA (either i.n. or i.v.).

4.3.5 Protection against challenge with VV-HA

Mice immunised with influenza virus are able to control infections with a recombinant VV expressing certain genes of influenza virus, with protection mediated by T lymphocytes (Doherty *et al.*, 1989). Mice immunised with 100 μg of pCMVIA/H1 were tested for their ability to control infection with a recombinant VV expressing the common HA antigen. Table 4.9 shows that challenge VV encoding HA (Coupar *et al.*, 1988) was recovered at much lower titres from mice that had been immunised with pCMVIA/H1 than from those that had not been given NAV. On the contrary, challenge virus was recovered from both pCMVIA/H1-primed and unprimed mice at similar titres when the wild-type VV strain, VV-WR (Wokatsch, 1972), which does not express HA, was used. These results suggest that the protective immunity was HA-specific.

Taken together, the findings in Sections 4.3.4 and 4.3.5 demonstrate the efficacy of NAV in generating protective anti-HA immune responses to control infections with virulent pathogens.

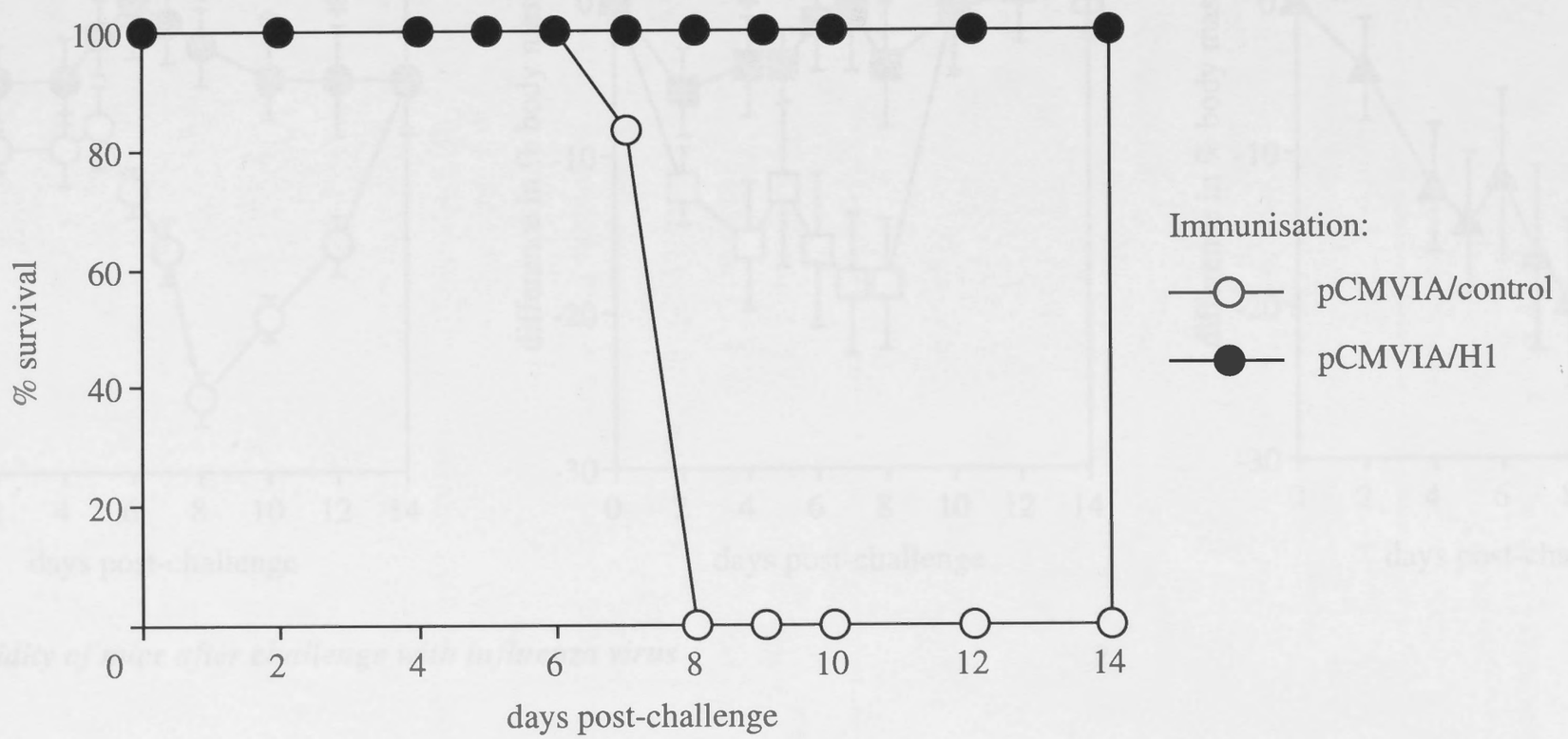


FIGURE 4.1. ***Mortality of mice after challenge with influenza virus***

Groups of 6 CBA/H mice were immunised with 100 μg of NAV i.m. At 4 wk post-immunisation, mice were challenged with 100 LD_{50} of influenza virus i.n. and were observed for mortality for 14 days. Similar findings were obtained in a duplicate experiment.

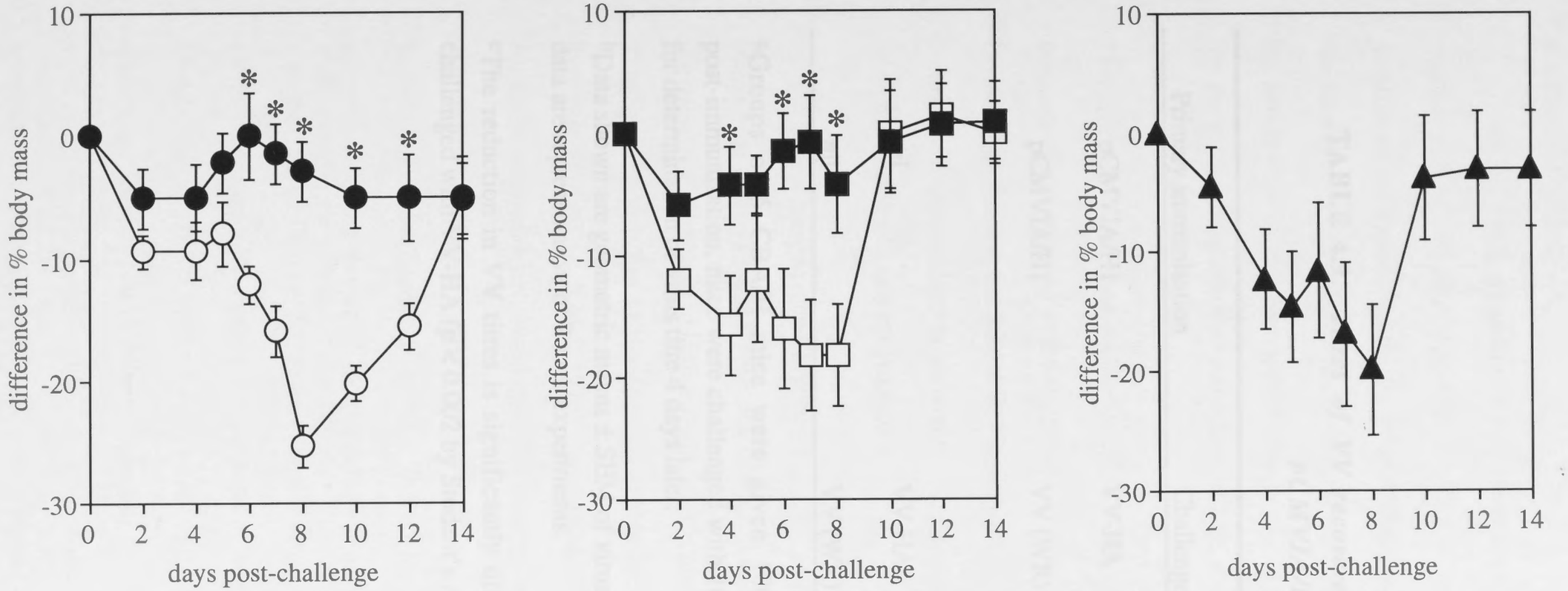


FIGURE 4.2. ***Morbidity of mice after challenge with influenza virus***

Groups of 6 CBA/H mice were immunised with (○) 100 μg of pCMVIA/control and 10^7 PFU of FPV-HA i.v., (●) 100 μg of pCMVIA/H1 and 10^7 PFU of FPV-HA i.v., (□) 100 μg of pCMVIA/control and 10^7 PFU of FPV-HA i.n., and (■) 100 μg of pCMVIA/H1 and 10^7 PFU of FPV-HA i.n. Boosting with FPV-HA was performed after 4 wk immunisation with NAV i.m. Mice were challenged with 100 LD_{50} of mouse-adapted influenza virus 1 wk after boosting. Mice immunised with (▲) 100 μg of pCMVIA/H1 alone were challenged at 4 wk post-immunisation. Morbidity was recorded as differences in percentage of body mass (Andrew and Coupar, 1988; Ulmer *et al.*, 1993) on different days post-challenge compared to original body mass on day 0. Data shown are the mean ± SEM of percentage of mass lost for 6 mice after challenge. The results are representative of 2 experiments. *$p < 0.05$, detemined by Student's *t* test in comparison with mice given pCMVIA/control before boosting.

TABLE 4.9. ***Titres of VV recovered from mice immunised with pCMVIA/H1***[a]

Primary immunisation	Challenge	Virus titres ($log_{10} \pm SEM$)[b]
pCMVIA/H1	VV-HA	4.92 ± 0.65[c]
pCMVIA/H1	VV (WR)	7.99 ± 0.13
nil	VV-HA	7.94 ± 0.16
nil	VV (WR)	8.20 ± 0.06

[a]Groups of 5 CBA/H mice were given 100 µg of pCMVIA/H1 i.m. At 2 wk post-immunisation, mice were challenged with 10^7 PFU of VV i.v. Ovaries were removed for determination of virus titre 4 days later.

[b]Data shown are geometric means ± SEM of virus titres in ovaries of 5 individual mice. The data are representative of 2 such experiments.

[c]The reduction in VV titres is significantly different in comparison to unprimed mice challenged with VV-HA ($p < 0.002$ by Student's *t* test).

4.4 DISCUSSION

In Chapter 3, recombinant FPV encoding cytokine genes were studied as potential vaccine vectors. Cytokines co-expressed by these recombinants were used to selectively manipulate the immune responses generated towards co-expressed vaccine antigen. NAV has also been reported to be an effective and potentially safe vaccine, evoking prolonged but low level immune responses (Fynan *et al.*, 1993; Ulmer *et al.*, 1993). In this chapter, the generation of protective immunity by i.m. inocula of NAV and the enhancement of these responses by subsequent immunisation with recombinant FPV was investigated. The strategy was based on the premise that immunisation with NAV produces low amounts of antigen, and in theory, should induce high affinity specific immune responses. On the other hand, recombinant FPV expresses higher levels of vaccine antigen that might be expected to considerably expand these responses. A consecutive vaccination strategy was designed to test this proposition.

This strategy proved highly successful, in that following sequential immunisation, both systemic and mucosal antibody responses were greatly elevated after boosting at the relevant site. Antibody responses induced by consecutive immunisation were higher than those elicited by either HA-encoding NAV or recombinant FPV vector alone. Levels of systemic antibody achieved using this strategy were not only higher than anamnestic antibody responses generated by immunisation with recombinant FPV (Chapter 3) but also higher than those found in convalescent sera (Kavaler *et al.*, 1991; Dr. H.L. Robinson, personal communication). These elevated antibody levels afforded enhanced protection against mortality and morbidity upon lethal challenge with influenza virus. As was the case in Chapter 3, these findings illustrate the importance of specific antibody in conferring immunity against homologous influenza virus strains as reported elsewhere (Askonas *et al.*, 1982). The capacity to prime for mucosal immunity via a systemic route, demonstrated here and by others (Fynan *et al.*, 1993; Montgomery *et al.*, 1993; Ulmer *et al.*, 1993), is a particularly attractive feature of NAV immunisation. Examples of mucosal immunity induced via systemic routes are scarce (Liang *et al.*, 1988), indeed mucosal immunity is usually generated by local vaccination (McGhee and Kiyono, 1993).

The demonstration that immunisation with NAV primes for mucosal immunity may offer an alternative strategy, besides recombinant FPV (Chapter 3), for immunoprophylaxis at mucosae. The capacity to boost local immune responses by the consecutive immunisation strategy described using NAV and recombinant FPV offers prospects for enhanced mucosal immunoprophylaxis.

The effects of cytokines encoded in recombinant FPV on boosting the mucosal antibody responses generated by this consecutive immunisation strategy were also examined. Interestingly, the suppression of antibody responses generated against the co-expressed HA antigen by FPV-expressed IFN-γ, as described in Chapter 3, was not observed in this experiment. Instead, boosting with FPV-HA-IFNγ augmented antibody responses generated by NAV. The effects of IFN-γ in inhibiting Th2 responses during the early development of immune responses (Belosevic *et al.*, 1989; Scott, 1991) may provide an explanation for this finding. During recall antibody responses, FPV-derived IFN-γ may have promoted IgG2a production established by NAV priming. This would be consistent with the induction of the IgG2a isotype by IFN-γ reported elsewhere (Snapper and Paul, 1987; Finkelman *et al.*, 1990).

Greater levels of recall mucosal antibody responses were elicited when IL-6 was co-expressed in recombinant FPV used for boosting after priming with NAV. The development of mucosal antibody responses has been shown to be critically dependent on IL-6, as mice lacking the IL-6 gene mount negligible mucosal responses following challenge with certain antigens (Ramsay *et al.*, 1994a). These responses are restored in deficient mice, however, following infection with recombinant VV expressing IL-6. In normal mice, IL-6 delivered to the mucosae by recombinant FPV elicited markedly higher antibody responses and primed for elevated recall antibody responses (Chapter 3). The present finding is a further demonstration of the role that IL-6 can play in the expansion of immune cells, particularly at the mucosae, during primary and secondary antibody responses. Again, strategies for the delivery of IL-6 could have major benefits in the development of improved local immunoprophylaxis.

An interesting finding was the selective induction of antibodies of the IgG2a subclass, a Th1 cytokine-driven immune response, after i.m. immunisation with NAV. A dramatic increase in the amount of IgG2a antibody usually follows infection of mice with replicating viruses, whereas infection with killed or inactivated viruses usually induces IgG1 antibodies (Nguyen *et al.*, 1994). It is unclear how i.m. immunisation with NAV selectively induced IgG2a antibodies, given that there is no evidence to suggest that these plasmids replicate in mammalian cells (Wolff *et al.*, 1992). A possible explanation is that IFN-γ produced by activated macrophages plays an important role in promoting IgG2a differentiation (Snapper and Paul, 1987; Finkelman *et al.*, 1990). When plasmid vectors are delivered by gene-gun through the skin, as opposed to the i.m. route used here, the predominant antibody response is of IgG1 subclass, a Th2 cytokine-driven response (Dr. H.L. Robinson, personal communication). Why this should differ from the response induced by i.m. inoculation is unclear. Activation of APC in the skin, such as Langerhans cells and keratinocytes (Moll, 1993; Nickoloff and Turka, 1994; Tapia *et al.*, 1994), to secrete IL-4, which in turn, promotes IgG1 production (Snapper *et al.*, 1988; Kuhn *et al.*, 1991; Goodman *et al.*, 1993) may be a possible explanation. However, a comparison of CTL responses between intradermal (i.d.) and i.m. inoculations revealed similar levels of CTL induction, suggesting that i.d. inoculation did not totally switch the response to a Th2 type with concomitant down-regulation of cell-mediated immunity (Dr. H.L. Robinson, personal communication). To date, it appears that the major isotype of specific antibodies elicited by NAV is governed by the site of inoculation. The selective induction of IgG2a antibodies following i.m. injection of NAV may be beneficial to the host, as this subclass is more effective than IgG1 at fixing complement and Fc receptor binding, and thus in promoting destruction of virus-infected cells by cell lysis and ADCC (Heusser *et al.*, 1977; Klaus *et al.*, 1979; Fanger *et al.*, 1989).

Although antibody responses were elicited after immunisation with NAV and were markedly augmented by boosting with recombinant FPV, these approaches were ineffective for the induction of specific primary CTL activity. However, strong secondary responses could be generated following restimulation of splenocytes *in vitro,* indicating

that anti-HA CTL precursors were generated after primary NAV immunisation. The induction of CTL responses in mice immunised with NAV encoding the influenza virus NP has also been shown after *in vitro* restimulation of splenocytes with class I MHC-restricted peptide epitopes of the NP (Ulmer *et al.*, 1993). In the present study, in order to demonstrate that CTL activity was generated *in vivo*, a model system was developed in which NAV-immunised mice were challenged with a recombinant VV expressing the common HA. A similar model was previously described by Doherty *et al.* (1989), in which it was shown that mice primed with influenza virus were protected from intracerebral challenge with a recombinant VV expressing the influenza virus NP. In this case, protective immunity was mediated by T lymphocytes. Data presented from the VV-challenge model, described in this chapter, reveal that NAV primes HA-specific immune responses sufficiently to afford good protection against VV-HA. Although T lymphocytes were not shown to be the effector cells involved in protection, the abovementioned experiments by Doherty *et al.* (1989) have shown that protection against NP-expressing VV was mediated by T lymphocytes. Nevertheless, specific antibodies induced by NAV immunisation may also be involved in mediating antiviral protection through ADCC. Given that CTL precursors were produced after NAV immunisation, it is unclear why boosting with recombinant FPV did not augment secondary CTL responses *in vivo*. It is possible that the extremely high antibody responses generated following consecutive immunisation with HA-expressing NAV and recombinant FPV may have inhibited the development of cell-mediated responses (Parish, 1972; Ramshaw *et al.*, 1976). In any case, when taken together, these findings not only verify the generation of CTL activity after NAV vaccination but also the efficacy of NAV in conferring immunity against infection with a virulent pathogen.

The aim of vaccination is to establish a safe and effective method of immunisation that will provide long-lasting immunity (Ada, 1991). The data presented here emphasise the effectiveness of NAV as a delivery vehicle for vaccine antigens to generate protective immunity. The immune response is directed towards the encoded antigens in the absence of antigenic competition with the vector, as was the case with recombinant FPV

(Chapter 3). The absence of a significant immune response to the NAV, together with the longevity of muscle cells, may provide an explanation for the persistence of NAV following inoculation and for the prolonged immune responses that are induced. In addition, multiple immunisations are likely to be more effective where there is a lack of primary immune responsiveness to the vector itself; indeed, successful revaccination with NAV (Flynn *et al.*, 1993; Ulmer *et al.*, 1993) as well as recombinant FPV (Chapter 3) has been demonstrated. Different vectors encoding the same vaccine antigen may also be used to enhance the efficacy of multiple immunisations, a strategy successfully illustrated in this chapter.

In summary, these results demonstrate the ability of NAV to induce both CTL and antibody responses. The levels of antibody were augmented by subsequent immunisations with recombinant FPV. The consecutive strategy illustrated here has implications for the development of vaccines, notably in situations in which other strategies have given poor immune responses.

CHAPTER 5

The effects of virus-encoded interleukin 7 on antiviral immunity

5.1 INTRODUCTION

IL-7, a stromal cell-derived cytokine, has pleiotropic effects on lymphoid cells of both B and T lineage, NK cells and monocytes. This cytokine was first described as a growth factor for precursor B lymphocytes *in vitro* (Namen *et al.*, 1988a and 1988b) while subsequent studies have shown that it may play important roles in the development of pre-T as well as mature T-lineage cells (Morrissey *et al.*, 1989; Murray *et al.*, 1989; Welch *et al.*, 1989; Varma *et al.*, 1990; Tushinski *et al.*, 1991). IL-7 also enhances the induction of CTL (Alderson *et al.*, 1990; Hickman *et al.*, 1990; Kos and Müllbacher, 1992), LAK cell (Alderson *et al.*, 1990; Hickman *et al.*, 1990; Lynch and Miller, 1990; Naume and Espevik, 1991; Stotter *et al.*, 1991; Kos and Müllbacher, 1992) and NK cell (Naume and Espevik, 1991) activities *in vitro*. In addition, IL-7 induces monocytes to secretion of IL-1α, IL-1β, IL-6 and TNF-α, and to tumouricidal activity (Alderson *et al.*, 1991).

Most of this information was obtained from *in vitro* investigations and relatively little is known about the *in vivo* activities of IL-7. Studies using mice in which IL-7 is expressed as a transgene (Samaridis *et al.*, 1991) and in mice lacking the IL-7 receptor gene (Maraskovsky *et al.*, 1993) have shown IL-7 to be an important regulatory factor in the development of B lymphocyte precursors, mature B lymphocytes, thymocytes and peripheral T lymphocytes. Administration of recombinant IL-7 to mice increases the numbers of precursor B lymphocytes in the bone marrow as well as mature B lymphocytes, and to a lesser extent, T lymphocytes, in the spleen and lymph nodes (Morrissey *et al.*, 1991b). Such treatment also accelerated the rate of recovery of lymphocytes in mice pretreated with cyclophosphamide (Morrissey *et al.*, 1991a) and increased the number of immature cells of the myeloid lineage (Faltynek *et al.*, 1992). The numbers of $CD8^+$ lymphocytes were elevated in the spleens of recombinant IL-7-treated mice, thus altering $CD4^+/CD8^+$ ratios (Faltynek *et al.*, 1992; Komschlies *et al.*, 1994).

The interaction of IL-7 with other cytokines has not been extensively studied. *In vitro* proliferation of LAK cells induced by IL-7 has been reported both to be

dependent on IL-2 (Morrissey *et al.*, 1989; Okazaki *et al.*, 1989) and to occur independently of this factor (Naume and Espevik, 1991). IL-7-induced LAK activity could be inhibited with IL-4 *in vitro* (Stotter and Lotze, 1991), although others have failed to reproduce this finding (Alderson *et al.*, 1990). Moreover, IL-7 has been shown to promote the generation of primary and secondary CTL *in vitro* in the absence of IL-2 (Kos and Müllbacher, 1992; Kos and Müllbacher, 1993; Carini and Essex, 1994) although antibodies against IL-2 substantially, but not completely, inhibited the stimulatory effect of IL-7 on alloreactive CTL generation (Alderson *et al.*, 1990). These findings suggest that IL-7 may act both through IL-2 and independently of this factor in stimulating cell-mediated immunity.

Cytokines expressed by recombinant VV have been found to have profound effects on host immune responses to the vector or to co-expressed antigen. This approach has been used to elucidate functional roles of a number of different cytokines during antiviral immune responses *in vivo* (Ramshaw *et al.*, 1992; Ramsay and Kohonen-Corish, 1993; Ramsay *et al.*, 1994a and 1994b). In this chapter, *in vivo* effects of IL-7 expression on antiviral immune responses and the interactions of this factor with host-derived cytokines were studied.

5.2 MATERIALS AND METHODS

5.2.1 Mice

Female 6-8-week old athymic nude Swiss outbred mice, bred under SPF conditions, were obtained from the Animal Breeding Establishment of the John Curtin School of Medical Research. Mice lacking the IFN-γ-receptor (IFN-γR) were kindly provided by Prof. M. Aguet, University of Zurich, and maintained under SPF conditions. Euthymic CBA/H mice are described in Section 3.2.1.

5.2.2 Culture medium

As described in Section 2.2.1.

5.2.3 Cell lines

Murine L929 (H-2^k) and human 143B cell lines are described in Section 3.2.3. The YAC-1 (H-2^a) cell line, derived from the Moloney leukemia virus-induced lymphoma in the A/Sn mouse (Kiessling *et al.*, 1975), and P815 (H-2^d) cells, a mastocytoma line derived from the DBA/2 mouse (Dunn and Potter, 1957), were grown in F15 medium.

The HT-2 (Watson, 1979), B9 (Aarden *et al.*, 1987) and clone K cells (kindly provided by Dr. Phil Hodgkin, JCSMR), used for detection of IL-2, IL-6 and IL-7, respectively, were grown in MLC.

5.2.4 Viruses

VV were grown and purified according to standard techniques (Boyle and Coupar, 1986). VV-HA-TK^- (Andrew *et al.*, 1986) was used for the construction of the IL-7 recombinant VV while VV-HA (TK^+; Coupar *et al.*, 1988) was used as the control virus. VV stocks were prepared as described in Section 2.2.4.

5.2.5 Virus titration

As described in Section 4.2.9.

5.2.6 DNA manipulation and Southern blotting

As described in Section 2.2.5.

5.2.7 Construction of recombinant VV

VV recombinants were constructed by marker rescue of the TK gene as previously described (Boyle and Coupar, 1986) and plaque-purified under methotrexate selection (Panicali and Paoletti, 1982). Monolayers of 143B cells were infected with VV-HA-TK$^-$ at a moi of 0.01 for 1 h and then transfected with plasmids (20 μg/ml) in HEPES-buffered saline [HEPES (20 mM; pH 7.0)-saline, $CaCl_2$ (0.13 M) and calf thymus DNA (20 μg/ml)]. Recombinant VV (TK$^+$), which grows under methotrexate selection, was identified by dot-blot analysis and probing with ^{32}P-labelled DNA containing foreign genes. Plaque purification was repeated until the virus population was homogeneous, after which virus stocks were prepared in CV-1 cells. The presence of DNA inserts was further analysed by RE analysis and Southern blotting of viral DNA. Southern blotting and probing of DNA fragments were performed under high stringency (Section 2.2.5).

5.2.8 Preparation of VV DNA

VV DNA was prepared from stock grown in 143B cell monolayers for RE and Southern blot analyses. VV-infected cells (48 h) were suspended in hypotonic buffer [Tris (10 mM; pH 7.8) and KCl (12 mM)], frozen and thawed thrice, and the supernatant was harvested. Virus particles in supernatant were collected by centrifugation on a sucrose gradient [36% (w/v)] and digested with proteinase K (50 μg/ml; BM) in sarkosyl [1% (w/v); Sigma]. The digests were purified by phenol-chloroform (50%:50%, v/v) extraction and precipitated with ethanol.

5.2.9 Preparation of spleen cells

As described in Section 3.2.5.

5.2.10 Cytotoxicity assays

CTL assays were performed as described in Section 3.2.6. For LAK assays, the NK-resistant and LAK-sensitive cell line, P815, was used. YAC-1 cells that are sensitive

to NK-mediated cytotoxicity were used for NK assays. The incubation time for NK assays was 4 h.

CTL, NK and LAK activity is expressed as total lytic units per spleen (Coligan *et al.*, 1992). One lytic unit is defined as the fraction of initial culture giving rise to 30% specific lysis of the target cell population.

5.2.11 Antibody assays

As described in Section 3.2.7.

5.2.12 *In vivo* and *in vitro* antibody treatment

The monoclonal rat antibodies S4B6 (anti-IL-2; Mosmann *et al.*, 1986), D9 (anti-CD8; de St. Groth *et al.*, 1986), GK1.5 (anti-CD4; Dialynas *et al.*, 1983), XMG-1.2 (anti-IFN-γ; Cherwinski *et al.*, 1987) and GL113 (anti-β-galactosidase; kindly provided by Dr. John Abrams, DNAX Research Institute) were prepared as ascites. For *in vivo* depletion of cell subsets or cytokines, ascites were injected i.p., 0.5 mg of antibody/mouse, 1 day before virus infection, on the day of virus infection, and on days 1, 2, 3 and 5 post-infection.

For *in vitro* depletion of B lymphocytes, the tissue culture supernatant of the rat hybridoma J11d (Bruce *et al.*, 1981) was used as follows. Splenocytes (10^7/ml) were incubated in J11d (1:5, v/v in F15 medium) on ice for 30 min. Rabbit serum (1 ml; Low-Tox®-M Rabbit Complement, Cedarlane Laboratories Ltd., Canada) was then added as a source of complement and the mixture was incubated at 37°C for a further 30 min. Viable cells were recovered on a Histopaque (Sigma Diagnostics, MO, USA) density gradient. The antibody depletion process was then repeated and the cells were finally washed twice in MLC medium. The B lymphocyte-depleted population was adjusted to appropriate concentrations for assay.

5.2.13 Proliferation assays

T lymphocyte proliferation was measured after stimulation of spleen cells with anti-CD3 antibody (145-2C11; Leo *et al.*, 1987). 96-well U-bottom microtitre plates

(Linbro) were coated with antibody at 125 ng/well. Spleen cells were then cultured at 2 x 10^5 cells/well in 200 µl of MLC for 72 h at 37°C. Each well was pulsed with 1 µCi of [^{3}H]-thymidine (78 Ci/mmole; ICN Radiochemicals) for the last 6 h of culture. Cells were then harvested onto filter mats using a Skatron 96 well harvester (Pharmacia). Scintillation fluid (LKB Scintillation Products) was added and the [^{3}H]-thymidine uptake was measured in a Pharmacia liquid scintillation counter.

5.2.14 Cytokine assays

Spleen cells were stimulated with immobilised anti-CD3 antibody, as described for the proliferation assay in Section 5.2.13, however, these cells were cultured for only 24 h prior to harvest of supernatants for assay for cytokines. Supernatants of virus-infected cell cultures were prepared as described in Section 2.2.9.

IL-2, IL-6 and IL-7 were detected by [^{3}H]-thymidine incorporation, as outlined in Section 2.2.9, using the IL-2-dependent HT-2, IL-6-dependent B9 and IL-7-dependent clone K cell lines, respectively. Due to the slight responsiveness of HT-2 cells to IL-4, IL-2-specific proliferation was determined after neutralisation of IL-4 using rat anti-murine IL-4 antibodies (11B11; kindly provided by Dr. P. Hodgkin, JCSMR). As described in Section 2.2.9, IFN-γ, TNF-α and IL-4 were assayed by indirect ELISA using rat anti-murine IFN-γ (R4.6A2; Spitalny and Havell, 1984), hamster anti-murine TNF (TN3-19.12; Sheehan *et al.*, 1989) and anti-murine IL-4 (11B11), respectively, as capture antibodies. Bound IFN-γ and TNF-α were detected using polyclonal rabbit anti-murine IFN-γ and TNF-α antisera, respectively, and alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Silenus). Captured IL-4 was assayed using biotinylated anti-IL-4 antibody (BVD6) followed by streptavidin-conjugated alkaline phosphatase. The ELISA assays were developed and analysed as described (Section 2.2.9).

5.2.15 Flow cytometry

Cells were labelled with flourochrome-conjugated antibody, and/or biotinylated antibody followed by flourochrome-conjugated streptavidin, using standard procedures (Loken, 1986). Splenocytes (10^6 cells), depleted of erythrocytes by water lysis, were

pelleted by gentle centrifugation at 4°C and were resuspended in 50 µl of a diluted flourochrome-conjugated antibody (Silenus) in cold PBS. After 30 min on ice, the staining mixtures were diluted 50-fold with cold PBS and cells were harvested by centrifugation at 4°C. For double staining of cells with a second antibody, the procedure outlined above was repeated with a secondary antibody. Biotinylated antibodies were detected using flourochrome-conjugated streptavidin (Amersham International Plc., UK). Antibody and flourochrome-conjugated streptavidin were used at dilutions of 1:20 (v/v) and 1:400 (v/v), respectively. Cell samples were resuspended in 0.5 ml of cold PBS for flow cytometry.

Labelled samples were analysed on a FACS 440 Flow Cytometer (Becton Dickson Immunocytometry System, CA, USA). For each sample, 10,000 cells/event were collected. Gating of dead cells was performed using forward light scatter. Analysis of data was performed using the PC-LYSYS software version 1.0 (Becton Dickson Immunocytometry System). The percentage of positively stained cells was calculated by subtracting control cells not stained with flourochrome-conjugated antibodies from those stained with flourochrome-conjugated antibodies.

5.2.16 Cell composition in the spleens

Cell types and numbers were determined by light microscopy after cytocentrifugation [500 rpm for 10 min in a cytospin centrifuge (Shandon Southern, England)] of 1 x 10^5 spleen cells and differential staining with May-Grünwald-Giemsa stain as described (Dacie and Lewis, 1984; Gulbenkian *et al.*, 1990). About 400 leukocytes were counted on each slide. The cell types were identified according to Diggs *et al.*

5.2.17 Statistical analysis

As described in Section 2.2.11.

5.3 RESULTS

5.3.1 Construction of insertion vectors for VV

A recombinant VV insertion plasmid, pLR01, containing a murine IL-7 gene under the transcriptional control of an early/late VV promoter was constructed for insertion of the cytokine gene into the *Hin*dIII F region of the recombinant VV (Fig. 5.1). The murine IL-7 cDNA contained in plasmid pSRα296-muIL7 was kindly provided by Dr. N. Wrighton (DNAX Research Institute of Molecular and Cellular Biology Inc.). A *Pst*I fragment of pSRα296-muIL7, containing the IL-7 gene, was generated and subcloned into *Pst*I-digested pGEM-3Z (Promega Corporation, WI, USA) with *Bam*HI and *Hin*dIII at the 5' and 3' ends of the IL-7 DNA, respectively (data not shown). This *Bam*HI and *Hin*dIII IL-7 gene fragment was then force-cloned into *Bam*HI and *Hin*dIII-digested pPS7.5A (Coupar *et al.*, 1988), in front of the early/late P7.5 VV promoter, giving rise to pLR01.

5.3.2 Construction and testing of VV-HA-IL7

In vitro homologous recombination of pLR01 with VV-HA-TK$^-$ gave rise to VV-HA-IL7, which contained the genes for IL-7 and HSV TK in the *Hin*dIII F region and the HA gene of influenza virus A/PR/8 gene in the J region. The genome arrangements of wild-type and recombinant VV are shown in Fig. 5.2.

The presence of the IL-7 insert was confirmed by RE analysis and Southern blotting. When digested with *Hin*dIII, VV-HA-TK$^-$ releases an F-fragment of ~13 kb (Panicali and Paoletti, 1982; Goebel *et al.*, 1990). Due to an *Hin*dIII site present in the recombinant F-fragment (Figs. 5.1 and 5.2), *Hin*dIII digestion of this fragment from VV-HA-IL7 gives rise to 2 fragments of ~9 kb and ~ 5 kb (Fig. 5.3). Next, the expression of biologically active IL-7 was tested by assay of supernatants from IL-7 VV-infected 143B monolayers using clone K cells. The resultant dose-dependent proliferation of clone K cells confirmed the production of IL-7 by cells infected with VV-HA-IL7 (Fig. 5.4).

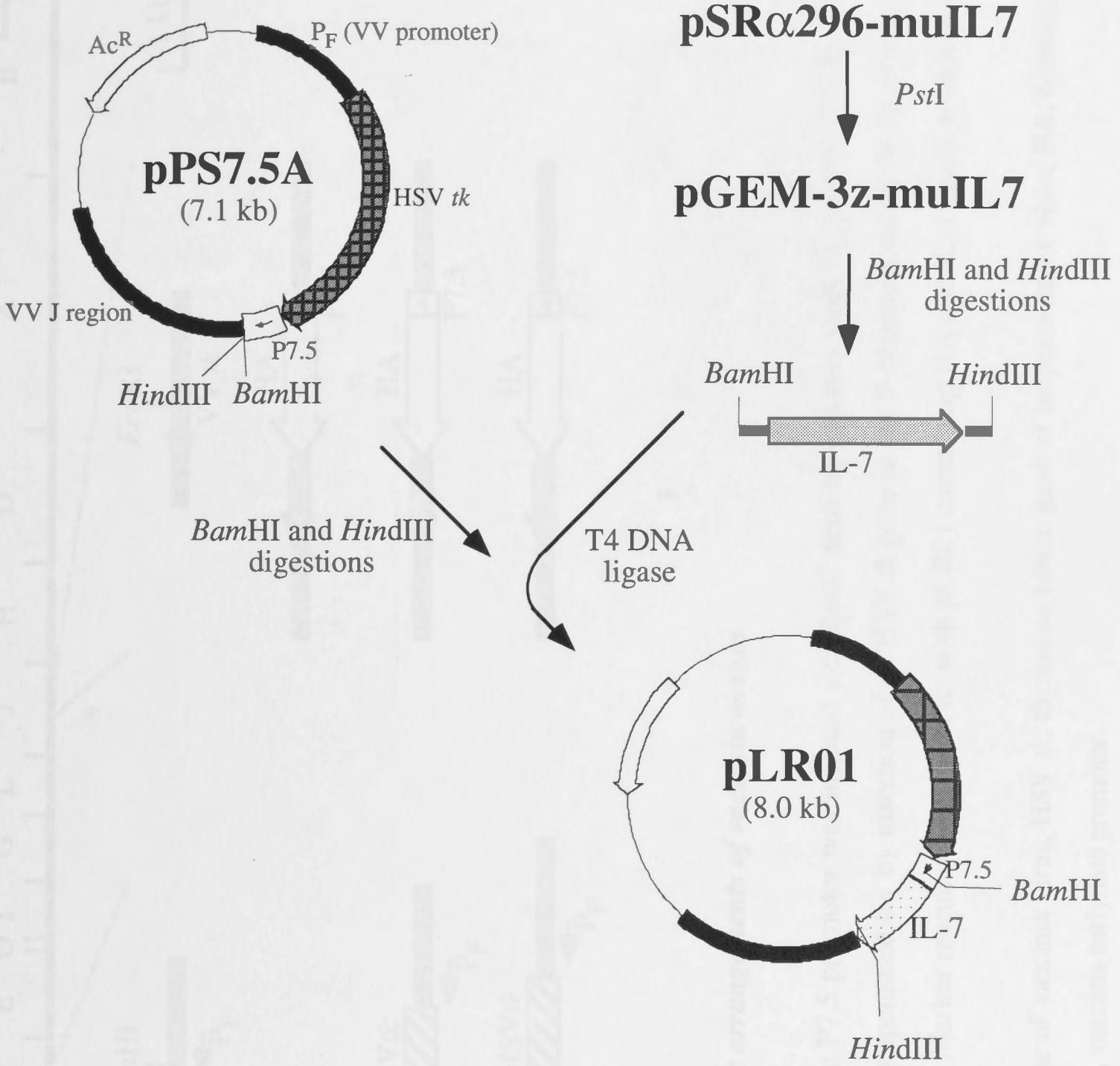


FIGURE 5.1. ***Construction of insertion plasmid pLR01***

A *Pst*I fragment containing the IL-7 gene was obtained from pSRα296-muIL7. The 5' and 3' ends of this fragment were then modified to have *Bam*HI and *Hin*dIII, respectively (see Section 5.3.1). Plasmid pLR01 was constructed after insertion of the *Bam*HI and *Hin*dIII IL-7 gene fragment in front of a P7.5 promoter in pPS7.5A (Coupar *et al.*, 1988).

Ac^R, ampicilin-resistance gene; P_F, vaccinia promoter; HSV *tk*, thymidine kinase gene of herpes simplex virus; P7.5, vaccinia early/late promoter; VV J region, J-fragment of the vaccinia virus *Hin*dIII digests

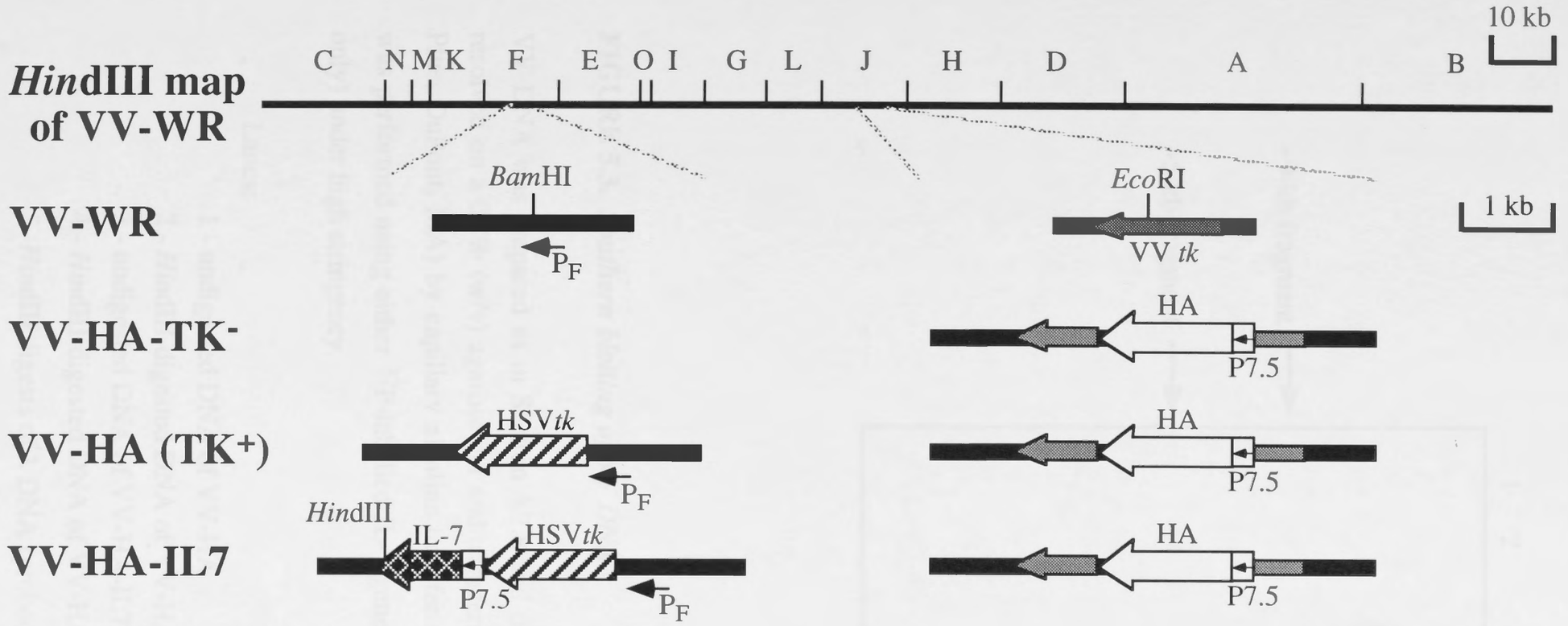


FIGURE 5.2. ***Schematic representation of the genomic arrangements of vaccinia viruses***

The HA gene of influenza virus, under the control of the P7.5 promoter was inserted into the *tk* gene of vaccinia virus in the *Hin*dIII J-fragment, yielding VV-HA-TK⁻. The disruption of the *tk* phenotype was compensated for by insertion of an HSV *tk* gene in the F-fragment under the P_F promoter. This give rise to VV-HA (TK⁺) which is used as the control virus. Marker rescue of VV-HA-TK⁻ with pLR01 containing HA and IL-7 genes produced VV-HA-IL7.

$\leftarrow P_F$, vaccinia promoter P_F; VV *tk*, thymidine kinase gene of vaccinia virus; HSV *tk*, thymidine kinase gene of herpes simplex virus; HA, hemagglutinin gene of influenza virus A/PR/8; IL-7, murine interleukin-7 gene; P7.5, vaccinia early/late promoter

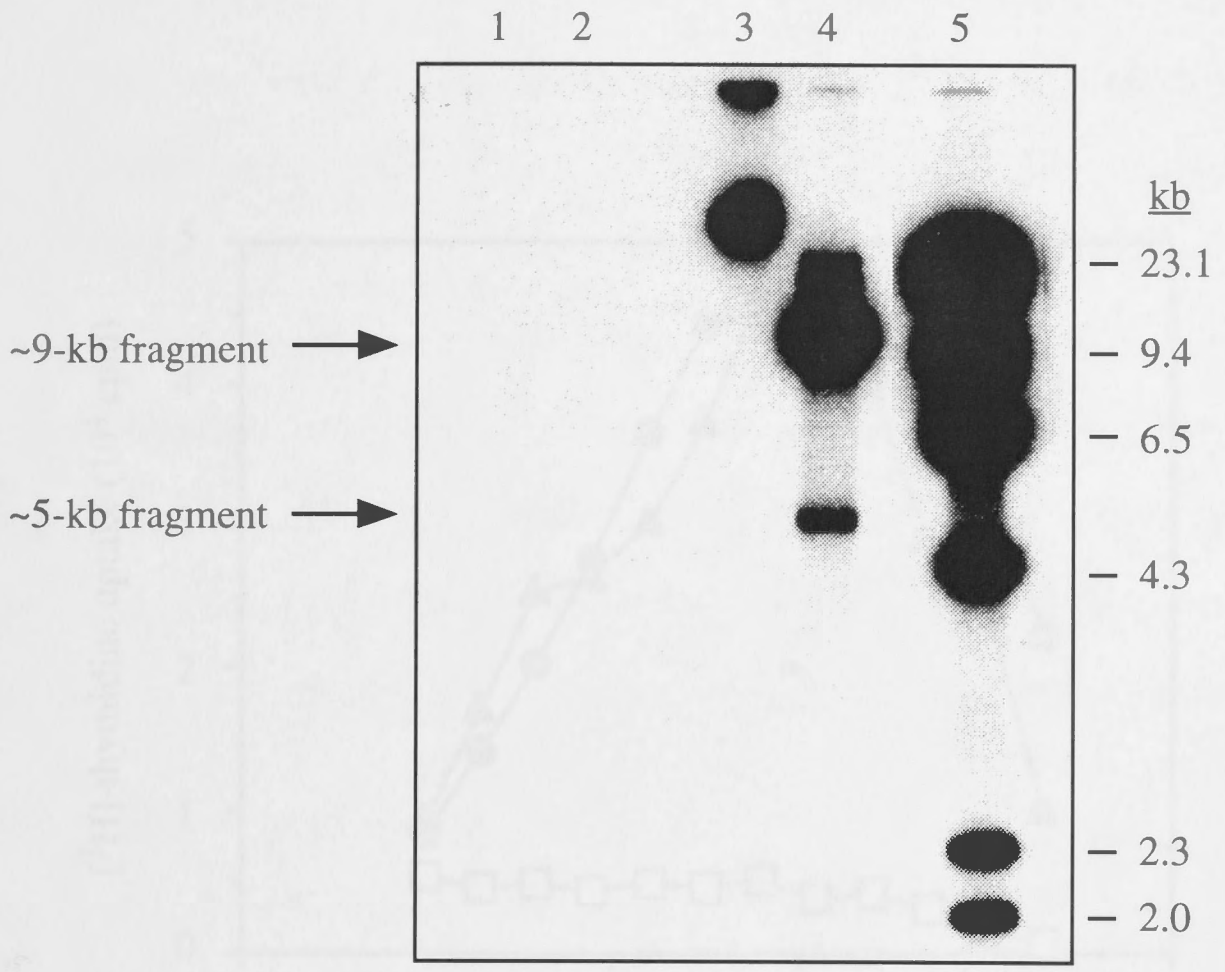


FIGURE 5.3. ***Southern blotting of VV DNA***

VV DNA was prepared as in Section 5.2.8 and digested with *Hin*dIII. The digests were resolved on a 0.7% (w/v) agarose gel and transferred onto nylon membranes (GeneScreen Plus, DuPont, MA) by capillary alkaline transfer (Sambrook *et al.*, 1989). Hybridisation was performed using either ^{32}P-labelled IL-7 gene or λ DNA probe (for λ *Hin*dIII digests only) under high stringency.

Lanes:

1 - undigested DNA of VV-HA

2 - *Hin*dIII-digested DNA of VV-HA

3 - undigested DNA of VV-HA-IL7

4 - *Hin*dIII-digested DNA of VV-HA-IL7

5 -*Hin*dIII digests of λ DNA (probed with ^{32}P-labelled λ DNA)

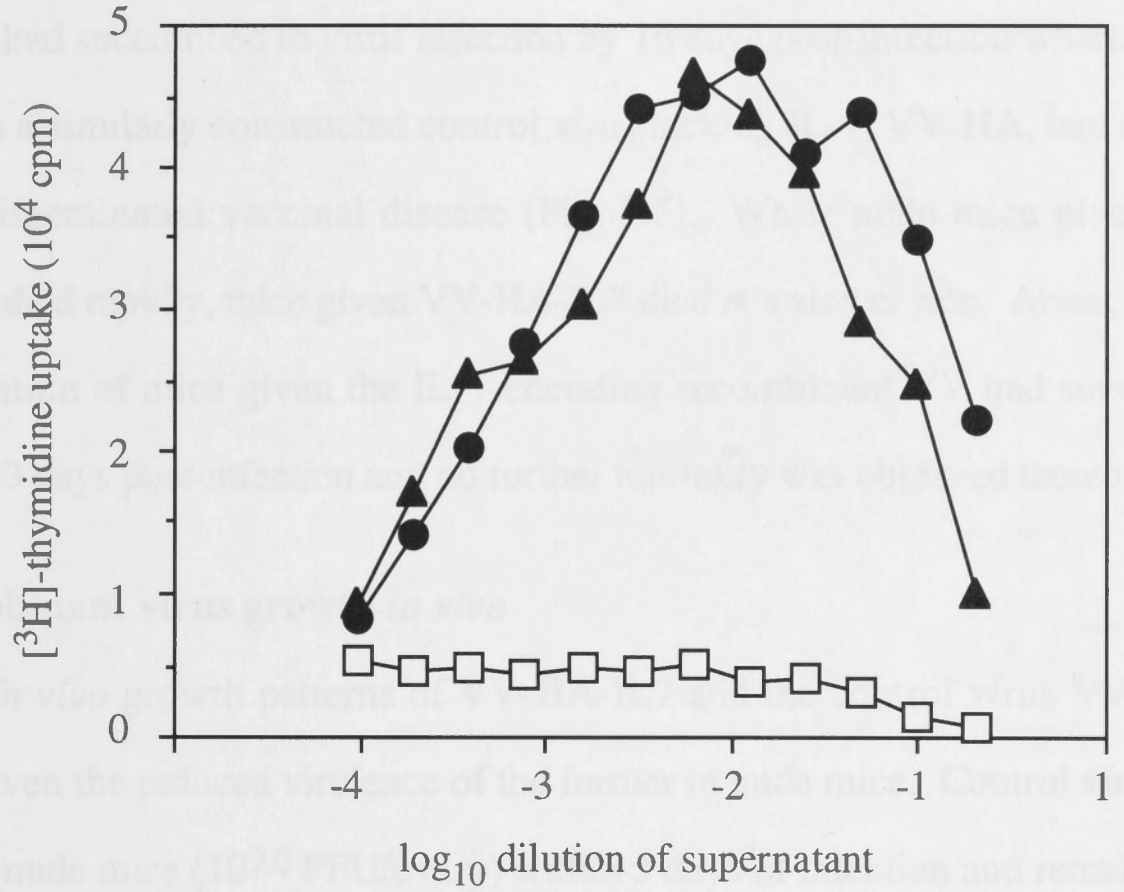


FIGURE 5.4. ***Production of murine IL-7 from recombinant VV-infected 143B cells***

Supernatants were taken from VV-HA-infected cells (□) and from those infected with VV-HA-IL7 for 8 h (▲) or 24 h (●), and diluted in microtiter before addition of 5 x 10^3 clone K cells per well. Results were expressed as mean of [^{3}H]-thymidine incorporation for triplicate supernatant samples over the last 6 h of a 48-h culture period. SEM were < 10% and are omitted for clarity.

5.3.3 Survival of immunodeficient mice

Immunodeficient athymic nude mice, lacking thymus-derived T lymphocytes, are highly susceptible to infection with VV. These mice were used to examine the effects of cytokine expression on virus attenuation (Ramshaw *et al.*, 1987; Karupiah *et al.*, 1990a and 1991; Ramshaw *et al.*, 1992). When given VV-HA-IL7 at 10^7 PFU i.v., only 13% of mice (n=23) had succumbed to virus infection by 16 days post-infection whereas all mice (n=10) given a similarly constructed control virus lacking IL-7, VV-HA, had died at this time from disseminated vaccinal disease (Fig. 5.5). While nude mice given VV-HA control virus died rapidly, mice given VV-HA-IL7 died at a slower rate. About 33% of the initial population of mice given the IL-7-encoding recombinant VV had survived virus infection at 60 days post-infection and no further mortality was observed thereafter.

5.3.4 Recombinant virus growth *in vivo*

The *in vivo* growth patterns of VV-HA-IL7 and the control virus VV-HA were compared, given the reduced virulence of the former in nude mice. Control virus grew to high titres in nude mice ($10^{9.0}$ PFU/ovary) within 3 days of infection and remained at this level until death (Table 5.1). However, the growth patterns of VV-HA-IL7 differed, reaching a peak titre of $10^{9.0}$ PFU/ovary at day 8 post-infection and declining thereafter (significantly different, $p < 0.05$ by Student's *t* test). These observations reveal that some immunodeficient mice began to resolve infection with recombinant VV expressing IL-7.

The attenuating effect of IL-7 expression was also seen in euthymic CBA/H mice. IL-7 expression induced an accelerated clearance of the virus. After reaching similar titres as the control virus at day 4 post-infection, VV-HA-IL7 was cleared by day 8 post-infection at which time the control virus could still be detected ($10^{4.85}$ PFU/ovary; Table 5.1).

Taken together, the findings in Sections 5.3.3 and 5.3.4 suggest that local production of vector-expressed IL-7 attenuated the growth of VV *in vivo*, probably through the induction of host responses. Possible mechanisms of this attenuation are studied below.

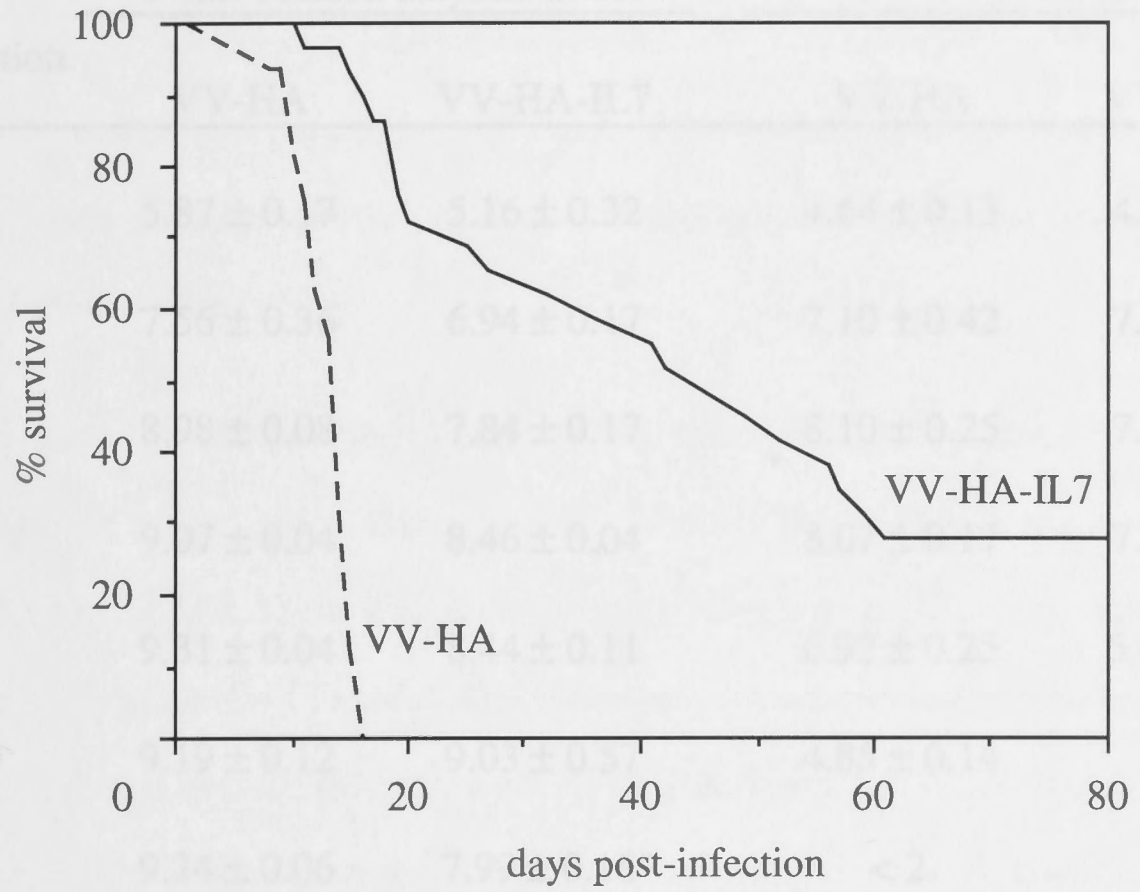


FIGURE 5.5. ***Survival of athymic Swiss outbred nude mice infected with VV recombinants***

Groups of 23 and 10 mice were given 10^7 PFU of VV-HA-IL7 and VV-HA i.v., respectively, and were observed for mortality for up to 80 days. Similar results were obtained in another experiment with 10 mice per group.

TABLE 5.1. ***Growth kinetics of recombinant VV in mice***[a]

Days post-infection	Log_{10} virus titers (mean of PFU/ovary ± SEM)[b]			
	Swiss outbred athymic nudes		CBA/H	
	VV-HA	VV-HA-IL7	VV-HA	VV-HA-IL7
1	5.87 ± 0.17	5.16 ± 0.32	4.64 ± 0.13	4.73 ± 0.09
2	7.56 ± 0.36	6.94 ± 0.17	7.10 ± 0.42	7.58 ± 0.21
3	8.98 ± 0.08	7.84 ± 0.17	8.10 ± 0.25	7.77 ± 0.16
4	9.07 ± 0.04	8.46 ± 0.04	8.07 ± 0.17	7.31 ± 0.23
6	9.31 ± 0.04	8.44 ± 0.11	6.92 ± 0.25	5.42 ± 0.19[c]
8	9.19 ± 0.12	9.03 ± 0.57	4.85 ± 0.14	< 2[c]
10	9.24 ± 0.06	7.99 ± 0.18[c]	< 2	< 2
14	8.19 ± 0.32[d]	7.07 ± 0.31[c]	ND[e]	ND
17	-	5.65 ± 0.91	ND	ND
20	-	5.12 ± 0.62	ND	ND

[a]Groups of 4 mice were given 10^7 PFU of recombinant VV i.v. and their ovaries were harvested on the days indicated. The virus load was estimated by plaque assay as described in Section 5.2.5. The limit of sensitivity of these assays was $> 10^2$ PFU/ovary.

[b]Each result represents the geometric mean ± SEM of virus titres in ovaries for 4 individual mice. These titres are representative of 2 experiments.

[c]Significantly different from mice infected with VV-HA ($p < 0.05$ by Student's *t* test).

[d]All nude mice given VV-HA eventually succumbed to virus infection.

[e]ND, not determined.

5.3.5 Induction of immune responses in mice given virus expressing IL-7

Next, the effects of recombinant VV-expressed IL-7 on antiviral immune responses were examined. After infection with 10^7 PFU of VV, there was an increase in splenic cellularity in mice given VV-HA-IL7 compared to those given control virus. Normal CBA/H mice infected with VV-HA-IL7 showed a 2-fold increase in their splenic leukocyte counts by 6 days post-infection (Table 5.2). The increased cellularity was even more pronounced in nude mice given VV-HA-IL7. Microscopic examination revealed a general increase in the proportion of different cell types in the spleens of CBA/H mice given VV-HA-IL7 (Table 5.3).

Spleen cells from infected mice were next tested for their cytolytic effector activity against different target cells. Using spleen effectors from CBA/H mice infected with either recombinant VV, there was no apparent difference in CTL lytic activity against VV-infected target cells (Table 5.4). However, when the considerable increase in splenocyte numbers in mice infected with VV-HA-IL7 was taken into account, peak CTL levels were 2-fold higher than in control virus-infected mice at 6 days after infection. Table 5.5 gives the CTL lytic activity per spleen in mice given either VV-HA-IL7 or control virus.

IL-7 is known to induce LAK (Alderson *et al.*, 1990; Hickman *et al.*, 1990; Lynch and Miller, 1990; Naume and Espevik, 1991; Stotter *et al.*, 1991; Kos and Müllbacher, 1992) and NK (Naume and Espevik, 1991) cell activities *in vitro*. For this reason, the NK and LAK lytic activity of splenocytes from mice given recombinant VV encoding IL-7 were also investigated. For this purpose, YAC-1 and P815 target cells, which have different sensitivities to NK lysis were used. YAC-1 cells are sensitive to NK lysis whereas P815 cells are NK-resistant. NK cells would, therefore, lyse only YAC-1 targets (Biron *et al.*, 1990). The induction of LAK activity would be revealed by lysis of both target cells (Li *et al.*, 1991).

Table 5.6 shows the lytic activity of spleen cells from euthymic mice against YAC-1 and P815 target cells. Splenocytes taken 3 days post-infection with VV-HA lysed

TABLE 5.2. ***Splenic cellularity in mice given recombinant VV[a]***

Mice	Days post-infection	Virus	Leukocytes (10^7)/spleen (mean ± SEM)[b]
CBA/H	3	VV-HA	5.8 ± 0.5
		VV-HA-IL7	4.5 ± 1.4
	6	VV-HA	9.2 ± 0.3
		VV-HA-IL7	16.5 ± 0.6[c]
	9	VV-HA	13.1 ± 2.3
		VV-HA-IL7	13.8 ± 2.7
Nude	3	VV-HA	7.5 ± 0.4
		VV-HA-IL7	14.8 ± 1.0[c]
	6	VV-HA	6.5 ± 0.3
		VV-HA-IL7	12.0 ± 1.2[c]
	9	VV-HA	3.9 ± 0.2
		VV-HA-IL7	16.5 ± 0.7[c]

[a]Groups of 4 mice were given 10^7 PFU of recombinant VV i.v. At the days indicated, spleens were harvested and splenocytes pooled. The number of leukocytes among the spleen cells were enumerated.

[b]Data shown are the mean ± SEM of leukocyte counts from 3 experiments.

[c]$p < 0.0006$ by Student's *t* test in comparison to mice given VV-HA.

TABLE 5.3. ***Cell composition in spleens of mice given recombinant VV[a]***

Leukocytes	Numbers of cells per spleen (10^7)[b]	
	VV-HA	VV-HA-IL7
Lymphocytes	6.9	11.8
Basophils	0.4	0.9
Neutrophils	0.4	0.9
Eosinophils	0.2	0.5
Monocytes	1.2	2.4

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant VV i.v. and spleens were removed 6 days after infection. The spleens were pooled and the cell composition among 1 x 10^5 spleen cells was determined as described in Section 5.2.16.

[b]A total of 400 spleen cells was enumerated and the proportion of different cells were determined following cytocentrifugation and staining by May-Grünwald-Giemsa. The total numbers of each cell type in the spleen was then calculated using the cell ratios obtained. Results are representative of 2 experiments.

TABLE 5.4. ***CTL activity of effectors from mice infected with recombinant VV[a]***

Days post-infection	Effector:target ratios	% specific lysis by effectors from mice infected with[b]:	
		VV-HA	VV-HA-IL7
3	60:1	24.14	32.86
	20:1	16.20	21.89
	6:1	8.64	5.54
	2:1	4.49	2.55
6	60:1	62.81	66.89
	20:1	49.68	58.97
	6:1	27.79	27.49
	2:1	8.58	9.23
9	60:1	38.89	47.61
	20:1	28.89	36.75
	6:1	11.54	17.95
	2:1	1.44	9.63

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant VV i.v. At the times indicated, spleens were removed and splenocytes pooled.

[b]Assays were performed using L929 target cells infected with VV (see Section 5.2.10). Data shown are means of triplicate wells and SEM were < 5%. Specific lysis of uninfected L929 cells was < 10%. These results are representative of 3 experiments.

TABLE 5.5. ***Kinetics of CTL responses in spleens of mice given recombinant VV***[a]

Days post-infection	Total lytic units per spleen in mice given (mean ± SEM)[b]:	
	VV-HA	VV-HA-IL7
3	89 ± 8	186 ± 58[c]
6	532 ± 17	1 145 ± 42[c]
9	198 ± 35	184 ± 36[d]

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant VV i.v. and spleens were removed at time points indicated. Antiviral CTL responses in pooled spleens against VV-infected L929 target cells were determined as described in Section 5.2.10.

[b]Each result represents the mean ± SEM of total lytic units at 30% specific lysis in triplicate wells. These results are representative of 3 experiments.

[c]$p < 0.05$ and [d]$p > 0.05$ by Student's *t* test. The differences of total lytic units detected in mice given VV-HA-IL7 on days 3 and 6 post-infection were significant when compared with mice infected with the control virus.

TABLE 5.6. ***Kinetics of NK and LAK activity in spleens of mice given recombinant VV[a]***

Mice	Targets	Days post-infection	Total lytic units per spleen in mice given (mean ± SEM)[b]:	
			VV-HA	VV-HA-IL7
CBA/H	YAC-1	3	38 ± 3	157 ± 49
		6	65 ± 2	143 ± 5
		9	67 ± 12	85 ± 17
	P815	3	150 ± 13	252 ± 78[c]
		6	46 ± 1	140 ± 5[c,d]
		9	60 ± 11	53 ± 10[c]
Nude	YAC-1	3	71 ± 4	182 ± 12
		6	65 ± 3	128 ± 14
		9	8 ± 1	32 ± 1
	P815	3	29 ± 2	157 ± 11[c,d]
		6	9 ± 1	137 ± 8[c,d]
		9	12 ± 1	45 ± 2[c,d]

[a]Groups of 4 mice were given 10^7 PFU of recombinant VV i.v. and spleens were removed on days indicated. NK and LAK activities of pooled splenocytes against YAC-1 or P815 cells were determined.

[b]Each result represents the mean ± SEM of total lytic units at 30% specific lysis in triplicate wells. Similar results were obtained a duplicate experiment.

[c]The lytic activities of splenocytes from mice infected with VV-HA-IL7 against P815 were not significantly different from YAC-1 lysis ($p > 0.6$ by Student's *t* test).

[d]The differences in P815 lysis among groups of mice infected with VV-HA-IL7 and control virus were significant ($p < 0.001$ by Student's *t* test).

P815 targets at greater levels than YAC-1 targets, however little difference was observed at days 6 and 9 post-infection. Hence, LAK responses were induced in mice given control virus and these were elevated, particularly at days 3 and 6 post-infection, in mice infected with the IL-7-expressing recombinant VV. These findings demonstrate an enhancement of LAK activity by VV-encoded IL-7 *in vivo*, in addition to the increased CTL activity shown above.

Previous reports have shown that nude mice mount antiviral NK responses to recombinant VV infection and that these responses are elevated by expression of IL-2 by the recombinant virus (Karupiah *et al.*, 1990b). Here, the effects of VV-encoded IL-7 on the generation of host immune responses to infection were also studied in athymic nude mice.

Table 5.6 also shows the lytic activity of splenocytes taken from VV-infected nude mice against YAC-1 and P815 target cells. There was greater lytic activity against YAC-1 cells compared to P815 targets mediated by splenocytes taken at 3 or 6 days after infection with VV-HA, thus confirming that NK responses were elicited by VV in nude mice. However, in mice given VV-HA-IL7, levels of P815 cell lysis by splenocytes taken at days 3 or 6 post-infection were as higher as those of YAC-1 targets. These levels of P815 lysis were higher than those observed in mice infected with control virus. These findings suggest that LAK responses were markedly elevated in nudes due to the expression of IL-7.

While these data suggest that virus-encoded IL-7 augmented CTL and LAK responses *in vivo*, the levels of specific antibody responses in CBA/H mice were not greatly enhanced by VV-expressed IL-7 (Table 5.7).

Together, these findings suggest that VV-encoded IL-7 selectively enhances cell-mediated immune responses *in vivo*.

TABLE 5.7. ***Systemic anti-VV IgG responses***[a]

Time post-infection (wk)	Reciprocal of end-point IgG titres in mice infected with[b]:	
	VV-HA	VV-HA-IL7
1	51 200	102 400
2	51 200	102 400

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant VV i.v. and anti-VV antibody responses were determined at time intervals as indicated.

[b]Results shown are the mean of reciprocal end-point titres for sera taken from 4 individual mice. The titres for 4 sera were the same and hence there were no SEM. Results are representative of 2 experiments.

5.3.6 Proliferation and cytokine secretion of splenocytes *in vitro*

The proliferation of spleen cells taken from VV-infected euthymic mice in response to stimulation with anti-CD3 antibody and the types of cytokines that they secrete following *in vitro* stimulation were compared. This experiment was performed because recombinant IL-7 has been shown to enhance proliferative responses of lymphoid cells (Komschlies *et al.*, 1994) and their secretion of cytokines *in vitro* (Armitage *et al.*, 1992b). Six days after infection with recombinant VV, at the peak of CTL responses, splenocytes from CBA/H mice were stimulated with plate-bound anti-CD3 antibodies. Splenocytes taken from mice infected with VV-HA-IL7 exhibited significantly higher rates of proliferation ($11.9 \pm 1.0 \times 10^4$ cpm; significantly different, $p < 0.01$ by Student's *t* test) than those taken from VV-HA-infected mice ($7.3 \pm 1.2 \times 10^4$ cpm). In addition, supernatants from the former contained greater levels of IL-2 and IL-6 than the control group (Table 5.8; $p < 0.05$ by Student's *t* test). No significant differences in the levels of IL-4, TNF or IFN-γ were noted.

Others have shown that IL-7 supports extrathymic differentiation of $\alpha\beta^+$ T lymphocytes in nude mice (Kenai *et al.*, 1993). Therefore, the effects of VV-encoded IL-7 on immune cells in nude mice were investigated. Splenocytes from nude mice given VV-HA-IL7 14 days earlier showed greater proliferation ($4.0 \pm 0.3 \times 10^4$ cpm; significantly different, $p < 0.001$ by Student's *t* test) in response to stimulation with plate-bound anti-CD3 antibody than splenocytes taken from mice infected with VV-HA ($1.1 \pm 0.1 \times 10^4$ cpm). Besides secreting more IL-2 and IL-6, splenocytes from VV-HA-IL7-infected nude mice also produced higher levels of IFN-γ than those taken from control virus-infected animals (Table 5.8).

Hence, it appears that expression of virus-encoded IL-7 enhanced responsiveness to CD3 stimulation and increased secretion of IL-2, IL-6 (nude and CBA/H mice), and IFN-γ (nude mice only) by splenocytes.

TABLE 5.8. ***Cytokine production by splenocytes after*** **in vitro** ***restimulation***[a]

Cytokine	Quantity of cytokine (U/ml) produced by splenocytes in mice given recombinant VV[b]:							
	CBA/H				Swiss outbred nude			
	Non-stimulated		Anti-CD3 stimulated		Non-stimulated		Anti-CD3 stimulated	
	VV-HA	VV-HA-IL7	VV-HA	VV-HA-IL7	VV-HA	VV-HA-IL7	VV-HA	VV-HA-IL7
IL-2	< 2	< 4	8	40	< 10	10	8	32
IL-4	< 2	< 2	32	46	< 2	< 2	< 2	4
IL-6	< 5	20	640	2560	40	80	200	3 950
TNF	10	10	20	20	< 2	< 2	< 2	< 2
IFN-γ	< 5	< 5	4 080	4 080	< 2	4	256	2 058

[a]Groups of 4 mice were given 10^7 PFU of recombinant VV i.v. and spleens were removed on day 6 (CBA/H mice) or 14 (nude mice) post-infection. Spleens within each group were pooled and B lymphocytes were depleted. After 24 h restimulation with plate-bound anti-CD3 antibody, supernatants were harvested and tested for the presence of different cytokines.

[b]The means of cytokine levels (units/ml) in triplicate wells are shown. SEM were < 10% and are omitted for clarity.

5.3.7 *In vivo* interactions between virus-expressed IL-7 and host cytokines

Given the findings that virus-encoded IL-7 alters the cytokine secretion pattern of splenocytes stimulated *in vitro*, it was of interest to study interactions of virus-encoded IL-7 with endogenously produced factors *in vivo*. Mice depleted of IL-2 or IFN-γ by treatment with monoclonal antibodies were infected with VV-HA-IL7 and the effect of the IL-2 or IFN-γ deficiency was studied. The influence of IFN-γ was also examined using mice with targeted disruption of their genes for the IFN-γR (IFN-γR-GKO).

Treating normal or nude mice with anti-IFN-γ antibodies had no effect on the increased splenic cellularity induced by VV-HA-IL7 (data not shown). IFN-γR-GKO mice also had similar numbers of spleen cells as wild-type control mice after infection with the IL-7-expressing recombinant VV (data not shown). In addition, there was no difference in lytic activity of spleen cells from these mice against P815 or VV-infected L929 target cells. Table 5.9 shows the effects of antibodies to IFN-γ on the levels of CTL and LAK activity in VV-HA-IL7-infected mice. Although these responses did not appear to be affected by abrogation of IFN-γ activity, both normal and nude mice infected with VV-HA-IL7 displayed signs of morbidity (ruffled fur, hunched posture) that were not seen in the control mice.

In contrast, treatment with anti-IL-2 monoclonal antibody reduced the number of splenocytes in VV-HA-IL7-infected CBA/H and nude mice to levels similar to those found in spleens of mice infected with VV-HA (data not shown). Accordingly, peak CTL or LAK responses generated in CBA/H mice 6 days after infection with VV-HA-IL7, expressed as total lytic units, were reduced to levels comparable to those elicited in mice given control virus (Table 5.9). In nude mice treated with anti-IL2 antibodies, splenic LAK responses (day 3 post-infection) were also reduced to control levels. Moreover, the effects of such depletion in nude mice were more dramatic than in CBA/H mice. All nude mice (n=6) given VV-HA-IL7 and depleted of IL-2 developed generalised vaccinal disease and died within 8-10 days (data not shown).

TABLE 5.9. *Effects of antibodies to IL-2 or IFN-γ on CTL and LAK activity in mice given recombinant VV*[a]

	Total lytic units per spleen in mice after antibody treatment (mean ± SEM)[b]:		
	GL113	XMG-1.2	S4B6
Day 6 anti-VV CTL responses[c] in CBA/H mice given:			
VV-HA	582 ± 19	612 ± 24	671 ± 58
VV-HA-IL7	1 049 ± 38	1 132 ± 47[d]	536 ± 167[e]
Day 6 LAK activity[f] in CBA/H mice given:			
VV-HA	55 ± 2	62 ± 7	47 ± 4
VV-HA-IL7	156 ± 6	174 ± 6[d]	63 ± 20[e]
Day 3 LAK activity[f] in athymic nude mice given:			
VV-HA	35 ± 2	38 ± 1	26 ± 1
VV-HA-IL7	142 ± 10	154 ± 9[d]	39 ± 4[e]

TABLE 5.9 (continued)

[a]Groups of 4 mice were given 10^7 PFU of recombinant VV i.v. and spleens were removed at times indicated. LAK and antiviral CTL activity in pooled spleens were determined as described in Section 5.2.10.

[b]Antibody treatments were performed as described in Section 5.2.12. Data shown are the means ± SEM of total lytic units at 30% specific lysis in triplicate wells. These results are representative of 2 experiments.

[c]Anti-VV CTL responses were determined by lysis of VV-infected L929 targets (see Section 5.2.10).

[d]The differences after treatment with antibodies to IFN-γ, XMG-1.2, are not significant ($p > 0.1$ by Student's *t* tests).

[e]The reduction of lytic activity after treatment with S4B6 (anti-IL-2) was significantly different in comparison to treatment with control GL113 ($p < 0.05$ by Student's *t* test).

[f]LAK activity was measured as described in Section 5.2.10.

These results clearly indicate that the attenuating effects of IL-7 on the growth of VV are mediated by host IL-2.

5.3.8 Effects of virus-encoded IL-7 on $CD4^+$ and $CD8^+$ lymphocytes, and the consequences of cytokine depletion

The influence of VV-encoded IL-7 on priming for $CD4^+$ and $CD8^+$ lymphocytes in the spleens of VV-infected CBA/H mice was examined by flow cytometry. There was a significant increase in the number of $CD4^+$ or $CD8^+$ lymphocytes in spleens of mice infected with VV-HA-IL7 over those infected with control virus, although the percentages of these lymphocytes were relatively similar (Table 5.10). The $CD8^+$ lymphocytes were shown to mediate antiviral cytotoxic activity, as abolition of CTL activity occurred following *in vivo* depletion of $CD8^+$ lymphocytes by anti-$CD8^+$ antibodies (data not shown).

Next, the involvement of host IL-2 and IFN-γ in generating increased numbers of $CD4^+$ and $CD8^+$ splenocytes was determined. Increases in both $CD4^+$ and $CD8^+$ lymphocyte numbers in mice infected with IL-7-expressing recombinant VV were blocked following administration of antibodies against IL-2 (Table 5.10). No such effect was observed, however, in anti-IFN-γ antibody-treated mice (Table 5.10) or in IFN-γR-GKO mice (data not shown).

These results reveal that virus-encoded IL-7 augments both $CD8^+$ and $CD4^+$ numbers in an IL-2-dependent but IFN-γ-independent manner.

TABLE 5.10. ***Numbers of CD4+ and CD8+ cells in spleens of mice given recombinant VV[a]***

Virus	Antibody treatment[b]	Total numbers x10^6/spleen [Percentage of cells] (mean ± SEM)[c]	
		CD4+	CD8+
VV-HA	GL113	4.50 ± 0.22 [10.4 ± 0.5]	6.80 ± 1.08 [15.7 ± 2.5]
	S4B6	4.24 ± 0.37 [10.4 ± 0.9]	6.49 ± 0.57 [15.9 ± 1.4]
	XMG-1.2	5.10 ± 0.21 [11.2 ± 0.7]	7.31 ± 0.87 [16.4 ± 1.2]
VV-HA-IL7	GL113	12.04 ± 1.33 [13.5 ± 1.5]	18.91 ± 3.39[d] [21.2 ± 3.8][e]
	S4B6	5.89 ± 1.83 [12.4 ± 3.9]	7.79 ± 2.42[e] [16.4 ± 5.1][e]
	XMG-1.2	11.12 ± 1.49 [12.9 ± 2.0]	20.21 ± 3.76[d] [18.6 ± 2.7][e]

[a]Groups of 4 CBA/H mice were given 10^7 PFU of recombinant VV i.v. and spleens were removed on day 6 post-infection. Splenocytes from pooled spleens were stained with PE-conjugated anti-mouse L3T4 (CD4; Becton Dickson Immunocytometry System, CA, USA) or biotin-conjugated anti-mouse Lyt-2 (CD8; Becton Dickson Immunocytometry System). Cell surface-bound anti-CD8 antibodies were detected using FITC- or PE-conjugated streptavidin (Amersham International Plc., UK). These cells were analysed by FACScan as described in Section 5.2.15.

[b]Antibody treatments were performed as described in Section 5.2.12.

[c]Each result represents the mean ± SEM of triplicate samples from the pooled spleens. Results are representative of 2 experiments.

[d]Significantly different in comparison to mice infected with control virus ($p < 0.03$ by Student's *t* test).

[e]Not significantly different in comparison to mice infected with control virus ($p > 0.05$ by Student's *t* test).

5.4 DISCUSSION

The expression of cytokine genes by recombinant VV has previously been shown to markedly alter the pathogenecity of the virus (Ramshaw *et al.*, 1992). Local production of encoded cytokines by recombinant VV at sites of infection has allowed studies of the immunobiology of these factors during antiviral immunity. While the expression of IL-4 enhanced viral pathogenicity (Sharma *et al.*, 1995), VV-encoded IFN-γ or TNF exhibited direct antiviral activities (Kohonen-Corish *et al.*, 1990; Sambhi *et al.*, 1991) and VV-encoded IL-2 stimulated an antiviral host immune response dependent on IFN-γ (Karupiah *et al.*, 1990b and 1991). Here, the attenuation of recombinant VV in normal and immunodeficient mice by expression of another factor, IL-7, is reported. The influence of this cytokine on host immune responses to VV infection was investigated.

Virus-encoded IL-7 dramatically increased the cellularity of the spleens of normal and nude mice. Splenomegaly or splenic leukocytosis has also been reported in mice given recombinant human IL-7 (Faltynek *et al.*, 1992; Komschlies *et al.*, 1994). While recombinant human IL-7 preferentially elevated splenic $CD8^+$ lymphocyte numbers (Faltynek *et al.*, 1992; Komschlies *et al.*, 1994), the present experiments have shown a generalised increase in both $CD4^+$ and $CD8^+$ lymphocyte numbers in normal euthymic mice. These findings may have been due to the activity of IL-7 as a cell growth factor. IL-7 has been shown to stimulate the development of both pre-T lymphocytes as well as mature cells of the T lineage (Morrissey *et al.*, 1989; Murray *et al.*, 1989; Welch *et al.*, 1989; Varma *et al.*, 1990; Tushinski *et al.*, 1991). Vector-derived IL-7 may also have prevented death of splenic cells by apoptosis. Cytokines, such as IL-2, have been demonstrated to prevent apoptosis (Pericle *et al.*, 1994) and may prevent activated immune cells from progressing to apoptosis (Salmon *et al.*, 1994). IL-7 has been reported to prevent apoptosis of dendritic epidermal T lymphocytes (Matsue *et al.*, 1993) and TNF-mediated apoptosis of mouse thymocytes upon stimulation with PHA (Hernández-Caselles and Stutman, 1993). Very recently, IL-7 expression during cell-cell interaction through integrins was shown to prevent apoptosis of leukemic cells from patients with chronic B-lymphocytic leukemia (Long *et al.*, 1995).

Along with a general proliferation of spleen cells, total splenic CTL and LAK activities were augmented in mice given VV-HA-IL7 compared to control virus-infected mice. The induction of enhanced antiviral CTL responses conforms with the reported *in vitro* actions of this cytokine in stimulating CTL proliferation (Alderson *et al.*, 1990; Lynch and Miller, 1990; Kos and Müllbacher, 1992). Given that $CD8^+$ CTL play an important role in resolving poxvirus infections (Blanden, 1971 and 1974), it is likely that the augmentation in numbers of these cells has contributed to the accelerated clearance of VV-HA-IL7 from infected mice.

The elevated LAK activity stimulated in nude mice by vector-encoded IL-7 may also have accelerated clearance of the virus, given that LAK cells have been demonstrated to have antiviral activity, particularly in immunodeficient animals (Bukowski *et al.*, 1988). Infection of nude mice with VV-HA-IL2 resulted in activation of NK activity that could resolve infection within 6-8 days (Karupiah *et al.*, 1990b and 1991). Although human IL-7 has been shown to stimulate NK cells *in vitro* (Naume and Espevik, 1991), murine IL-7 encoded by VV preferentially stimulated LAK activity *in vivo* in this study. This finding disagrees with the reported absence of LAK activity in mice given recombinant human IL-7 and challenged with tumour cells (Komschlies *et al.*, 1994). The inability of human IL-7 to induce LAK activity in mice may be due to species specificity of the cytokine. Enhancement of *in vitro* LAK activity by IL-7, however, has been widely reported (Alderson *et al.*, 1990; Hickman *et al.*, 1990; Lynch and Miller, 1990; Naume and Espevik, 1991; Stotter *et al.*, 1991; Kos and Müllbacher, 1992). Furthermore, data obtained in this study also show that VV-HA-IL7 infections persisted for longer periods than those involving the IL-2-encoding VV, suggesting that the antiviral properties of LAK cells induced by vector-expressed IL-7 may not have been as effective as the antiviral effector functions of NK and NK-like cells elicited by virus-encoded IL-2 (Karupiah *et al.*, 1990b). Nevertheless, the ability to induce antiviral LAK activity *in vivo* by delivery of vector-expressed IL-7 in immunodeficient individuals, as described in this study, may be an important therapy for infections with some viruses, such as CMV (Bukowski *et al.*, 1988).

Vector-expressed IL-7 primed splenocytes from euthymic mice for enhanced and accelerated proliferative responses, and greater production of IL-2 and IL-6 following stimulation with anti-CD3 antibody *in vitro*. Others have also reported enhanced proliferative responses of splenocytes to various T lymphocyte stimuli, including con A and anti-CD3 monoclonal antibodies, in the presence of recombinant IL-7 (Komschlies *et al.*, 1994). Moreover, T lymphocytes stimulated with IL-7 *in vitro*, in the absence of co-mitogen, secreted IL-2, IL-4, IL-6 and IFN-γ when restimulated with phorbol ester and ionomycin (Armitage *et al.*, 1992b). It is interesting that IL-7 also primed splenocytes from nude mice to respond to anti-CD3 in the present study, with resultant IL-2, IL-6 and IFN-γ production, despite their supposed lack of thymus-derived T lymphocytes. VV-expressed IL-7 may have stimulated $\alpha\beta^+$ T lymphocytes, given that IL-7 has been reported to support extrathymic differentiation of $\alpha\beta^+$ T lymphocytes in nude mice (Kenai *et al.*, 1993). While it is possible that small numbers of these T lymphocytes may have been present due to the reported "leaky" nature of the *nu/nu* mutation (Kishihara *et al.*, 1987; MacDonald *et al.*, 1987; Payer *et al.*, 1992), it is also possible that IL-7 has expanded a small proportion of T lymphocytes of the γδ phenotype in nude mice. Such a population has been identified among peripheral blood $CD3^+$ T lymphocytes of immunodeficient humans (Borst *et al.*, 1987). These lymphocytes are extrathymic and derive from organs such as the spleen, skin, intestine, lymph nodes, lungs and liver (Haas *et al.*, 1993). In nude Balb/c mice, alloreactive T lymphocyte clones with γδ TCR have also been isolated after allogeneic stimulation (Bluestone *et al.*, 1988). Such $\gamma\delta^+$ T lymphocytes may have provided a source of IL-2 in nude mice infected with VV-HA-IL7, given that splenic and intestinal $\gamma\delta^+$ T lymphocytes have been shown to secrete cytokines, including IL-2 and IFN-γ, in response to anti-CD3 stimulation (Bluestone *et al.*, 1991). In addition, IL-7 has been shown to act on $\gamma\delta^+$ lymphocytes, together with IL-1, stimulating these lymphocytes to proliferation (Skeen and Ziegler, 1993), while cell lines expressing IL-7 have been shown to support the generation of $\gamma\delta^+$ T lymphocytes after injection into spleens of young athymic nude mice (Gutierrez and Palacios, 1991). Human T lymphocytes with γδ TCR have been shown to display cytotoxic effector activity (Borst *et al.*, 1987; Brenner *et al.*, 1987), while in mice, γδ T lymphocytes play a

role in immunity against influenza virus (Carding *et al.*, 1990; Eichelberger *et al.*, 1991) and HSV-1 (Johnson *et al.*, 1992) infections. In the light of these findings, it is possible that vector-encoded IL-7 stimulates antiviral LAK activity and cytokine production among $\gamma\delta^+$ T lymphocytes in nude mice. Attempts to measure increased levels of $\alpha\beta^+$ or $\gamma\delta^+$ T lymphocytes in nude mice given VV-HA-IL7 gave inconclusive results.

While IL-7 has been shown to induce the expression of IL-2 and IL-2 receptor by NK cells (Naume and Espevik, 1991) and T lymphocytes (Morrissey *et al.*, 1989), the interactions of IL-7 with IL-2, as well as with other cytokines, have not been clearly elucidated. The generation of CTL by IL-7 *in vitro* has been reported to occur independently of IL-2 (Kos and Müllbacher, 1992; Kos and Müllbacher, 1993; Carini and Essex, 1994), although IL-2-dependency has also been suggested (Alderson *et al.*, 1990). The data obtained in this study support the latter findings. The activity of vector-encoded IL-7 in enhancing CTL and LAK responses *in vivo* was critically dependent on host IL-2. Vector-expressed IL-7 primed splenocytes to IL-2 production, as shown upon *in vitro* restimulation with antibodies to CD3, and the resultant IL-2 appeared to be essential for the IL-7-mediated enhancement of cell-mediated immune responses. The augmented responses were apparently due to an IL-2-mediated increase in splenocyte numbers rather than a selective expansion of CTL. The dependency on host IL-2 for the attenuation of IL-7-encoding VV was more clearly shown in nude mice, where removal of IL-2 activity totally reversed the virus attenuation. In these mice, vector-expressed IL-7 may have induced LAK cells to secrete IL-2, given that LAK cells have been shown to be capable of secreting this factor and IFN-γ (Moretta *et al.*, 1986). Blockade of IFN-γ activity did not abrogate IL-7-mediated attenuation of VV infection, although there were signs of pathology in mice deficient of IFN-γ. This suggests that IFN-γ may have contributed some important antiviral functions. Previous studies using IFN-γ-expressing recombinant VV have demonstrated the direct antiviral activity of this factor (Kohonen-Corish *et al.*, 1990). Taking these findings together, it is possible that vector-expressed IL-7 may have stimulated CTL or LAK cells to produce IL-2, with resultant autocrine stimulation of

proliferation leading to increased CTL or LAK cell numbers and elevated immune responses.

The finding that VV-encoded IL-7 primed splenocytes for elevated IL-6 secretion is in agreement with previous reports. Murine peripheral blood T lymphocytes, when stimulated with con A *in vitro*, produced high levels of IL-6, while antibodies to IL-6 do not affect the ability of IL-7 to stimulate proliferation of T lymphocytes (Morrissey *et al.*, 1989). Given that IL-6 has many functions, including that of a growth and differentiation factor for CTL (Van Snick, 1990), it is possible that host-derived IL-6 induced by vector-expressed IL-7 may have acted to expand activated immune cells in an interaction similar to that described for IL-2. Greater insights into the interactions between IL-6 and IL-7 *in vivo* could be attained using mice lacking IL-6 functions either by treatment with specific antibodies or targeted disruption of the IL-6 gene.

It has previously been shown that IL-7 is a growth factor for activated B lymphocytes (Joshi and Choi, 1991). Although virus-expressed IL-7 was shown to have significant effects on the induction of antiviral cell-mediated immune responses, this factor had no major effects on the generation of specific antiviral antibody responses. This may be due to the production of virus-encoded IL-7 in sites other than those in the vicinity of activated B lymphocytes. It could also be, as previously suggested by Joshi and Choi (1991), that IL-7 may not induce activated B lymphocytes to IgG secretion. The adjuvant effect of IL-7 in preferentially inducing augmented cell-mediated responses may be important for the control of intracellular pathogens, such as that suggested for prophylactic and therapeutic strategies against HIV (Salk *et al.*, 1993). Indeed, such vaccine strategies might be implemented using recombinant FPV, a virus successfully used as a safe and effective vaccine vector in Chapter 3.

In summary, the expression of IL-7 by a recombinant VV attenuated the recombinant virus and selectively augmented cell-mediated responses in a manner critically dependent on host IL-2.

CHAPTER 6

General Discussion

This thesis describes the selective induction of immune responses by poxvirus-encoded cytokines and the development of novel immunisation strategies using recombinant FPV and NAV vectors either alone or in combination.

The profile of cytokines present during the development of immune responses may critically influence the type of response generated against a particular antigen (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989a; Sher and Coffman, 1992). Interleukin 2 and IFN-γ, produced by Th1 clones, promote cell-mediated immunity (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989a) whereas IL-4, IL-5 and IL-10, secreted by Th2 lymphocytes, direct antibody responses (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989a; Sher and Coffman, 1992). These Th1 and Th2 subsets also cross-regulate, i.e. IFN-γ inhibits Th2 lymphocyte proliferation (Fernandaz-Botran *et al.*, 1988; Gajewski and Fitch, 1988), while IL-4 and IL-10 down-regulate Th1 responses (Powrie and Coffman, 1993a and 1993b).

Cytokines expressed by recombinant VV *in vivo* have profound effects on immune responses elicited against the virus (Karupiah *et al.*, 1990b; Ramshaw *et al.*, 1992; Ramsay and Kohonen-Corish, 1993; Ramsay *et al.*, 1994a and 1994b). Local production of these VV-encoded cytokines, at sites of infection, has also provided an effective tool for *in vivo* investigations into the biology of the encoded cytokine. For example, IL-2, when expressed by a recombinant VV, dramatically attenuated the pathogenicity of the virus (Ramshaw *et al.*, 1987; Flexner *et al.*, 1987). The attenuating effect of IL-2 was due to the induction of NK or NK-like cells to produce IFN-γ (Karupiah *et al.*, 1990b and 1991). The authors concluded that host IFN-γ had direct antiviral activities. Handa *et al.* (1983) have previously reported activity of IL-2 on NK cells and IFN-γ production *in vitro*. The evidence for direct antiviral activity by IFN-γ came when Kohonen-Corish *et al.* (1990) showed that recombinant VV encoding IFN-γ was highly attenuated in immunodeficient athymic or γ-irradiated mice. Very recently, in agreement with reports that IL-4 promotes Th2 immune responses (Le Gros *et al.*, 1990; Sadick *et al.*, 1990; Swain *et al.*, 1990; Chatelain *et al.*, 1992; Hsieh *et al.*, 1992; Romani *et al.*, 1992; Seder *et al.*, 1992) and inhibits Th1 responses (Hsieh *et al.*, 1992;

Seder *et al.*, 1992; Powrie and Coffman, 1993a and 1993b; Powrie *et al.*, 1994), VV-encoded IL-4 was shown to enhance virus pathogenicity by suppressing the induction of antiviral CTL (Sharma *et al.*, 1995). This inhibition of CTL generation was associated with the down-regulation of IL-2, IFN-γ and IL-12, as determined by PCR.

In the present study, the immunobiology of IL-6 and IFN-γ encoded by recombinant FPV vectors and of IL-7 encoded by recombinant VV were examined, together with their influence on immune responses to vaccination. IL-6 is a strong signal for B lymphocyte differentiation (Vink *et al.*, 1988) and acts on terminally differentiated plasma cells (Kishimoto and Hirano, 1988). This factor may be particularly important for the development of mucosal antibody responses (Beagley *et al.*, 1989). Indeed, expression of IL-6 by a recombinant VV in mutant mice deficient for this factor has substantiated its pivotal role in the development of antibody responses at the mucosae (Ramsay *et al.*, 1994a). The influence of IL-6 in stimulating production of IgG1 antibody in other *in vivo* models (Suematsu *et al.*, 1989) was also observed in normal mice infected with IL-6-expressing recombinant VV (Ramshaw *et al.*, 1992). The activity of FPV-encoded IL-6, in the present study, confirms these observations. Moreover, results described in Chapters 3 and 4 have extended these findings and suggest that IL-6 may also expand lymphocyte populations during recall antibody responses. These findings provide further insight into the biology of IL-6 *in vivo* and also indicate that strategies for the delivery of IL-6, as described in this thesis, may have major benefits in immunoprophylaxis for enhanced antibody responses, particularly at the mucosae.

IFN-γ has been shown to directly inhibit the development of Th2 responses (Fernandaz-Botran *et al.*, 1988; Gajewski and Fitch, 1988); this inhibition occurs during the early phases of the immune response (Belosevic *et al.*, 1989; Scott, 1991). Mice infected with IFN-γ-expressing recombinant VV mounted poor systemic anti-VV antibody responses (Kohonen-Corish *et al.*, 1990). This suppression was even more pronounced when IFN-γ was encoded in recombinant FPV in the present study,

however there was no reduction in the development of antiviral cell-mediated immunity. The inhibition of antibody responses by IFN-γ appeared to occur early in the generation of immunity, as FPV-expressed IFN-γ had little effect in suppressing antibody responses already established by NAV immunisation (Chapter 4). Recently, IL-12 was shown to be an important early factor in IFN-γ production and establishing cell-mediated immunity (Gazzinelli *et al.*, 1993; Heinzel *et al.*, 1993; Hsieh *et al.*, 1993b; Manetti *et al.*, 1993 and 1994; Seder *et al.*, 1993; Sypek *et al.*, 1993; Sieling *et al.*, 1994). When given *in vivo*, IL-12 promoted Th1 responses, through elevation of IFN-γ production by NK cells or T lymphocytes (Gazzinelli *et al.*, 1993; Heinzel *et al.*, 1993; Sypek *et al.*, 1993). Encoding IL-12 into recombinant FPV or VV would allow a critical analysis of its role in immune regulation. It would be predicted that the cytokine would preferentially induce cell-mediated immune responses which may be critical in protection against certain pathogens.

IL-7 has been reported to elevate the generation of CTL (Alderson *et al.*, 1990; Hickman *et al.*, 1990; Kos and Müllbacher, 1992) and LAK (Alderson *et al.*, 1990; Hickman *et al.*, 1990; Lynch and Miller, 1990; Naume and Espevik, 1991; Stotter *et al.*, 1991; Kos and Müllbacher, 1992) activity *in vitro*. Little is known about the interaction of this cytokine with other immune modulators. Data in Chapter 5 reveal that IL-7, expressed by recombinant VV, selectively augmented CTL and LAK responses and that this enhancement was mediated through host IL-2. This dependency on IL-2 supports similar *in vitro* findings by Morrissey *et al.* (1989), Okazaki *et al.* (1989) and Alderson *et al.* (1990). The ability to selectively generate cell-mediated immune responses by the actions of IL-7 or IFN-γ may have important implications for the development of vaccines where immunisation for such responses may be essential for effective prophylaxis, such as against virus infections.

Data presented here reveal that *in vivo* activities elucidated by encoding cytokines in FPV not only agree with those obtained using VV expression, or by other *in vitro* and *in vivo* studies, but also gave further insights into their biology. Recombinant poxviruses, particularly FPV, should prove useful for further

investigations into the immunobiology of other cytokines, such as IL-4, IL-7, IL-12 and other co-stimulatory molecules, for example B7 and CD40L. As a vector, recombinant FPV offers several advantages over VV for this purpose, such as prolonged expression of encoded genes in infected cells and the lack of interference from endogenous virus-encoded proteins. This approach may also allow the studies of cytokine interactions, by the use of viruses harbouring multiple cytokine genes. The technology of local cytokine gene expression, described here, has recently been extended to other expression systems, namely bacteria, retrovirus-transformed tumour cells and other viruses. Expression of IL-4 by *Salmonella* inhibited macrophage killing and increased the pathogenicity of the recombinant bacteria (Denich *et al.*, 1993), while IL-1-β expression protected mice from γ-irradiation-induced death (Carrier *et al.*, 1992). Studies into the biology of cytokines expressed by recombinant cytomegaloviruses are now being attempted (W.H. Burns, The John Hopkins University, personal communication). Transformed tumour cells expressing cytokines have also been shown to generate antitumour responses in syngeneic recipients (Alosco *et al.*, 1993; Gansbacher *et al.*, 1990). Recently, cytokine genes encoded by recombinant plasmids, when delivered into mouse muscles, were shown to have profound effects on the immune responses generated against challenge antigens (Raz *et al.*, 1993).

The acceptability of any virus as a potential vaccine vector depends mainly on its safety and efficacy (Ada, 1991 and 1994). The use of conventional vaccines and some recombinant virus vectors, particularly VV, has certain inherent limitations in these respects. The most notorious side-effect is the occasional reversion of attenuated strains to virulent phenotypes giving rise to vaccine-induced or vaccine-associated complications (Lane *et al.*, 1969; Lane *et al.*, 1970; Behbehami, 1983; Norrby, 1987). The use of vaccine strategies described in this thesis may overcome these limitations and offer potentially safe approaches to vaccination.

The safety of recombinant FPV lies in the host-restriction of this virus to avian cells. FPV infection in mammalian cells is non-productive or abortive (Taylor and Paoletti, 1988). In this study, severely immunodeficient mice did not succumb when

given recombinant FPV (Chapter 3). Earlier, avipoxvirus vectors were shown to be non-pathogenic in test animals (Paoletti *et al.*, 1994) and well-tolerated in human volunteers (Cadoz *et al.*, 1992). Nevertheless, foreign genes under the transcriptional control of early poxvirus promoters are expressed during abortive infection, resulting in presentation of heterologous proteins to the immune system and induction of immune responses (Taylor *et al.*, 1988b; Cox *et al.*, 1993b).

Intramuscular immunisation with NAV offers another potentially useful vaccine strategy. Although safety considerations are still being investigated, the induction of antibodies against DNA (Jiao *et al.*, 1992) or integration of plasmids in the host genome (Wolff *et al.*, 1992) have not been demonstrated. NAV can also be delivered via other routes, such as i.d. and i.v. (Fynan *et al.*, 1993; Zhu *et al.*, 1993), however the particle bombardment ('gene-gun') method (Fynan *et al.*, 1993) may represent an optimal mode of NAV immunisation. Besides offering a practical and easy vaccine delivery site, i.e. the epidermis, the gene-gun method provides a direct and intracellular delivery of very small quantities of NAV (< 1 μg; Haynes *et al.*, 1994), all these are important considerations for the use of vaccines in developing countries. The use of the gene-gun for NAV delivery may also enable a cocktail of recombinant plasmids expressing different genes to be delivered into individual cells. In this way, cytokine-expressing NAV can be given together with NAV encoding vaccine antigens in attempts to modify immune responses elicited against the vaccine antigen. In any case, cheap, stable and potentially effective vaccines for priming immune responses may be established using novel, non-replicating NAV and recombinant FPV vectors.

Another concern pertaining to the use of VV-based vaccines is whether prior exposure to the immunising vehicle will prevent the induction of optimal immune responses upon revaccination (Rooney *et al.*, 1988; Cooney *et al.*, 1991; Etlinger and Altenburger, 1991). This concern arose, for example, when some volunteers for vaccination with a recombinant VV expressing gp160 of HIV were found to possess antibodies to VV (Cooney *et al.*, 1991), even though they were vaccinated against smallpox many years earlier during the Smallpox Eradication campaign (Fenner *et al.*,

1988). Pre-existing immunity from smallpox vaccination has been found to modulate immune responses to a novel antigen expressed by a recombinant VV (Rooney *et al.*, 1988; Cooney *et al.*, 1991; Etlinger and Altenburger, 1991). The demonstration that there is no immunological cross-reactivity between FPV and VV in Chapter 3 suggests that the use of FPV as a vaccine vector would overcome this problem. Moreover, VV infections may expose many endogenous virus-encoded antigens, besides the heterologous vaccine antigen, that will also stimulate the immune system. Antigen competition between such proteins may modulate immune responses generated against the vaccine antigen (Andrew *et al.*, 1986). The use of recombinant FPV and NAV, which do not produce vector-encoded proteins, may offer the further advantage of a reduction in antigenic competition between vector and vaccine proteins. The resultant poor immune response mounted against recombinant FPV or NAV vectors may delay their clearance and allow prolonged expression of the vaccine antigen. Indeed, in Chapter 3, recombinant FPV was shown to persist in immunised animals. Others have found that muscle cells retain plasmids for prolonged periods (Wolff *et al.*, 1992). Such persistence of vaccine vectors may account for the induction of long-lasting immunity in recipient animals, a feature observed in the present study.

In the event of reduced primary immune responses generated against the vector, booster immunisations are likely to be more effective. Accordingly, prior exposure to the vaccine vector did not inhibit boosting of immune responses by recombinant avipoxviruses, as shown in Chapter 3 and by Cadoz *et al.* (1992). Fynan *et al.* (1993) and Ulmer *et al.* (1993) have also demonstrated the feasibility of multiple immunisations using NAV for boosting. To further improve the efficacy of multiple immunisations, non-cross reactive vaccine vectors encoding the same vaccine antigen could be used (Li *et al.*, 1993). Such immunisation protocols, using recombinant NAV and FPV vectors, proved highly successful in Chapter 4 and may provide a potentially effective vaccination regime where previous approaches have given poor immune responses. One potential application using such a consecutive immunisation strategy would be the boosting of the low levels of immunity usually induced against gag-pol

antigens of HIV (Walker *et al.*, 1987). Cooney *et al.* (1993) have successfully elevated specific T lymphocyte and antibody responses by using sequential vaccination with recombinant VV expressing the env of HIV and boosting with recombinant gp160.

The efficacy of a vaccine also lies in its ability to generate an appropriate immune response, given that some pathogens are most effectively controlled by the induction of either cell-mediated or antibody responses (Scott and Kaufmann, 1991; Sher and Coffman, 1992; Sher *et al.*, 1992; Urban *et al.*, 1992). A correlation also exists between Th lymphocyte phenotype dominance and susceptibility in many diseases, such as in infections by *Leishmania* or HIV, toxoplasma or helminth infestations, or in tuberculosis and listeriosis. Furthermore, an uncontrolled or chronic Th lymphocyte response can also lead to the immunopathology seen in vernal conjunctivitis or exposure to allergens and during infections with respiratory syncytial virus or *Borrelia* (Wierrenga *et al.*, 1990; Maggi *et al.*, 1991; Parronchi *et al.*, 1991; Yssel *et al.*, 1991; Yssel *et al.*, 1992; Alwan *et al.*, 1994). Therefore, the ability to selectively generate an appropriate immune response to vaccination, as illustrated in this thesis, may not only offer advantages in improving prophylactic strategies, but may also allow modification of immune responses for a specific pathogen with minimum pathology during immunotherapy.

Several therapeutic vaccines against tuberculosis, and herpesvirus or hepatitis virus infections are currently under development (Cohen, 1994). In addition, immunotherapy to boost or modify the immune systems of individuals infected with HIV has lately begun to receive much attention. Although antibodies may be important for preventing initial infection by the virus, high levels of antibodies in seropositive individuals do not prevent progression to AIDS. For treatment of an established infection, the induction of cell-mediated responses may be more beneficial (Clerici *et al.*, 1992). It has been shown that IL-12 can restore lost HIV- and influenza virus-specific cell-mediated responses among peripheral mononuclear cells cultured from asymptomatic seropositives (Clerici *et al.*, 1993b), that IFN-γ inhibits HIV replication (Rosenberg and Fauci, 1991) and selectively induces cell-mediated

immunity (Chapter 3), and that IL-7 augments CTL responses (Chapter 5). On this basis, immunotherapy to boost cell-mediated immune responses might be achieved using vaccine vectors encoding IL-7, IL-12 or IFN-γ together with an HIV antigen. Similar approaches may also have considerable implications for HIV prophylaxis, in that the preferential generation of cell-mediated immune responses may be of paramount importance for successful vaccination against HIV (Salk *et al.*, 1993). There are several potential antigens of HIV that could be used in a vaccine, however perhaps the most suitable would be the gag-pol protein. This antigen has major CTL determinants and is highly conserved (Nixon *et al.*, 1988), as opposed to the highly divergent env of HIV (Siliciano *et al.*, 1988). Moreover, the loss of T lymphocyte function in AIDS appears to be related to the sensitivity of lymphocytes to apoptosis (Clerici *et al.*, 1994a). Stimulation of lymphocytes from HIV-infected individuals results in apoptosis which can be reversed with selected cytokines, such as IL-2. It has been shown that cytokines, such as IL-2, can prevent apoptosis of activated immune cells (Salmon *et al.*, 1994). IL-7 has also been reported to prevent apoptosis of dendritic epidermal T lymphocytes (Matsue *et al.*, 1993) and TNF-mediated apoptosis of mouse thymocytes upon stimulation with PHA (Hernández-Caselles and Stutman, 1993). Hence, the prevention of apoptosis by vector-directed delivery of cytokines, as described in this thesis, may be an important factor for HIV immunotherapy.

Besides the prophylactic and therapeutic ramifications for infectious disease, the ability to preferentially manipulate the immune system using vector-encoded cytokines may also be important for cancer treatment and prophylaxis. Many tumours are non-immunogenic, as their antigens are not presented to the immune system and due to a lack of costimulation of immune cells (Hewitt *et al.*, 1976). Recently, animals given tumour cells transfected with cytokine genes or co-stimulatory molecules have not only rejected these transfectants, but were also resistant to a subsequent challenge with the original tumour. Cytokines found to possess antitumour-inducing capacity include IL-2 (Fearon *et al.*, 1990), IL-4 (Tepper *et al.*, 1989), IL-7 (Lynch *et al.*, 1991; Komschlies *et al.*, 1994), IL-12 (Noguchi *et al.*, 1995), IFN-γ (Gansbacher *et al.*, 1990), TNF

(Blankenstein *et al.*, 1991) and GM-CSF (Colombo *et al.*, 1991). Activation molecules, such as B7, have also been found to possess antitumour activities, in that immunisation with tumour cells tranduced with B7 generated strong antitumour cell-mediated immunity in test animals (Baskar *et al.*, 1993). Transfections of tumour cells are usually performed *ex vivo* with recombinant retroviruses, however the vectors described in this study represent alternatives for the delivery of cytokines and/or other immune activation molecules for cancer therapy *in vivo*. Recently, the co-administration of recombinant IL-12 during p53 peptide vaccination has led to regression of established Meth A sarcoma in mice (Noguchi *et al.*, 1995). Recombinant FPV or NAV could be constructed to express tumour-specific or associated antigens, such as p53 and other intracellular antigens prevalent in tumour cells (Melief and Kast, 1993) together with selected cytokines, and used for cancer prophylaxis.

Studies using recombinant VV expressing cytokine genes to overcome deficiencies in mutant mice with targeted gene disruptions (Ramsay *et al.*, 1994a) point to another potential application for FPV or NAV vectors — gene therapy. Indeed, attempts using recombinant plasmids to complement defects in muscular dystrophy have already been initiated (Acsadi *et al.*, 1991a). Although successful expression of human factor VIII by recombinant adenovirus vectors may have important implications for gene therapy for haemophilia A (Connelly *et al.*, 1995), recombinant avipoxviruses and NAV are potentially superior to other vectors used in gene therapy because of their persistence and the protracted expression of encoded foreign genes in recipient animals. Indeed, NAV immunisation was shown to be more effective than recombinant adenoviruses or retroviruses for the delivery of antigen to muscle cells (Davis *et al.*, 1993).

In conclusion, vectors expressing cytokines represent an effective strategy that may be used to selectively manipulate the immune response to a variety of diseases including cancer, AIDS and other infections.

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APPENDICES

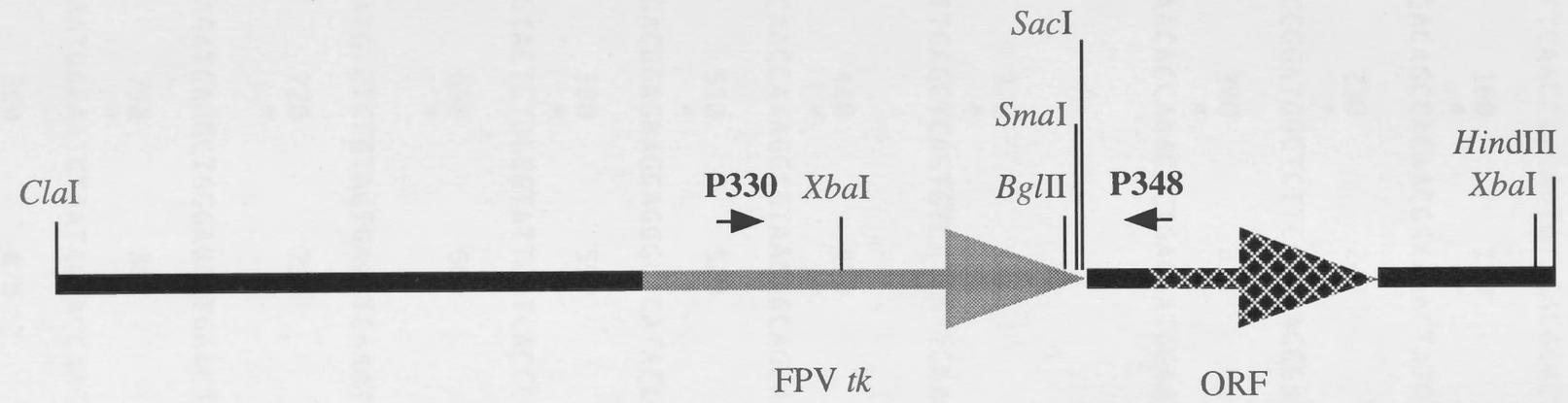
APPENDIX I

Sequences of primers used in this thesis

Primer	Sequences (5'....3')	Description
P330	TAGGTATAGACGAGGC	PCR analysis of recombinant FPV[a]
P348	ATCGAACTCCATTCCG	
PHA-1	ACTGGATCCATGAAGGCAAACCTA	PCR cloning of HA gene
PHA-2	ACTGGATCCAGATCTAAAAAAAATCAGATGCATATTCTGCACTGCAAAGATCC	
PIL6-1	ACTGGATCCATGAAGTTCCTCTCT	PCR cloning of IL-6 gene
PIL6-2	ACTGGATCCAAAAAAAACTAGGTTTGCCGAGTAGATCTCAAAGTGAC	
PIFNG-1	ACTGGATCCATGAACGCTACACACTGCATCTTGGCT	PCR cloning of IFN-γ gene
PIFNG-2	ACTGGATCCAAAAAAAATCAGCAGCGACTCCTTTTCCGCTTCCT	

[a]See also Appendix II

APPENDIX II



Location of primers P330 and P348 in the genome of fowlpox virus (FPV)

These primers were used to detect insertion of foreign genes at the single site down-stream of the *tk* gene. Under PCR conditions outlined in Section 2.2.5, a DNA fragment of about 0.4-kb long was produced after PCR with DNA prepared from FPV-M3 (wild-type virus). Insertion of foreign genes downstream of the *tk* gene in recombinant FPV generated PCR fragments of > 3-kb long.

ORF, open reading frame; *tk*, thymidine kinase gene

APPENDIX III

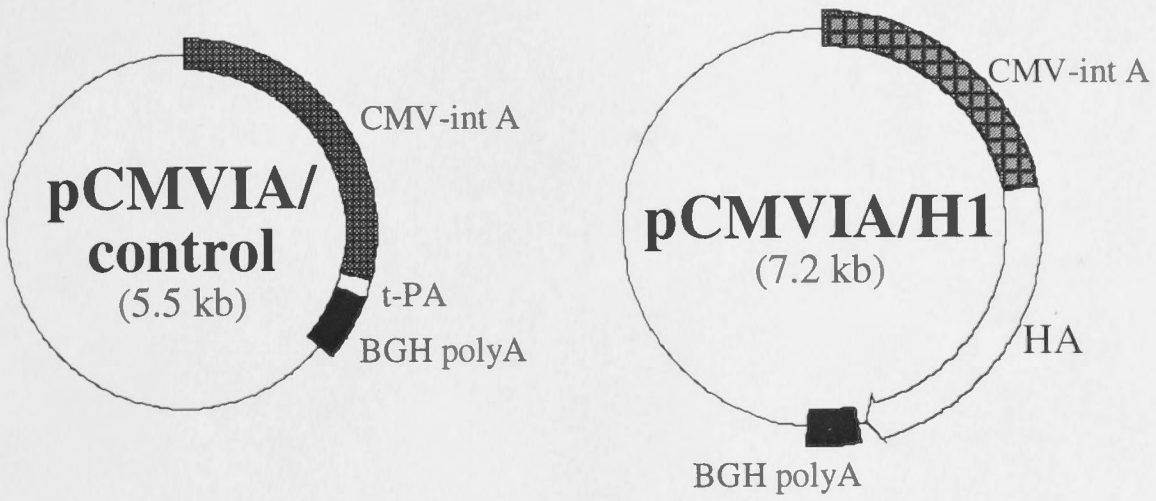
```
        10         20         30         40         50         60         70
         *          *          *          *          *          *          *
ATGAAGGCAAACCTACTGGTCCTGTTATGTGCACTTGCAGCTGCAGATGCAGACACAATATGTATAGGCT

        80         90        100        110        120        130        140
         *          *          *          *          *          *          *
ACCATGCGAACAATTCAACCGACACTGTTGACACAGTGCTCGAGAAGAATGTGACAGTGACACACTCTGT

       150        160        170        180        190        200        210
         *          *          *          *          *          *          *
TAACCTGCTCGAAGACAGCCACAACGGAAAACTATGTAGATTAAAAGGAATAGCCCCACTACAATTGGGG

       220        230        240        250        260        270        280
         *          *          *          *          *          *          *
AAATGTAACATCGCCGGATGGCTCTTGGGAAACCCAGAATGCGACCCACTGCTTCCAGTGAGATCATGGT

       290        300        310        320        330        340        350
         *          *          *          *          *          *          *
CCTACATTGTAGAAACACCAAACTCTGAGAATGGAATATGTTATCCAGGAGATTTCATCGACTATGAGGA
                                                                 P100 ➔

       360        370        380        390        400        410        420
         *          *          *          *          *          *          *
GCTGAGGGAGCAATTGAGCTCAGTGTCATCATTCGAAAGATTCGAAATATTTCCCAAAGAAAGCTCATGG

       430        440        450        460        470        480        490
         *          *          *          *          *          *          *
CCCAACCACAACACAACCAAAGGAGTAACGGCAGCATGCTCCCATGCGGGGAAAAGCAGTTTTTACAGAA

       500        510        520        530        540        550        560
         *          *          *          *          *          *          *
ATTTGCTATGGCTGACGGAGAAGGAGGGCTCATACCCAAAGCTGAAAAATTCTTATGTGAACAAGAAAGG

       570        580        590        600        610        620        630
         *          *          *          *          *          *          *
GAAAGAAGTCCTTGTACTGTGGGGTATTCATCACCCGTCTAACAGTAAGGATCAACAGAATATCTATCAG

       640        650        660        670        680        690        700
         *          *          *          *          *          *          *

AATGAAAATGCTTATGTCTCTGTAGTGACTTCAAATTATAACAGGAGATTTACCCCGGAAATAGCAGAAA

       710        720        730        740        750        760        770
         *          *          *          *          *          *          *

GACCCAAAGTAAGAGATCAAGCTGGGAGGATGAACTATTACTGGACCTTGCTAAAACCCGGAGACACAAT

       780        790        800        810        820        830        840
         *          *          *          *          *          *          *
AATATTTGAGGCAAATGGAAATCTAATAGCACCAAGGTATGCTTTCGCACTGAGTAGAGGCTTTGGGTCC

       850        860        870        880        890        900        910
         *          *          *          *          *          *          *
GGCATCATCACCTCAAACGCATCAATGCATGAGTGTAACACGAAGTGTCAAACACCCCTGGGAGCTATAA
                  ➔ P200

       920        930        940        950        960        970        980
         *          *          *          *          *          *          *
ACAGCAGTCTCCCTTTCCAGAATATACACCCAGTCACAATAGGAGAGTGCCCAAAATACGTCAGGAGTGC
```

```
       990       1000       1010       1020       1030       1040       1050
         *          *          *          *          *          *          *
CAAATTGAGGATGGTTACAGGACTAAGGAACATTCCGTCCATTCAATCCAGAGGTCTATTTGGAGCCATT

      1060       1070       1080       1090       1100       1110       1120
         *          *          *          *          *          *          *
GCCGGTTTTATTGAAGGGGGATGGACTGGAATGATAGATGGATGGTACGGTTATCATCATCAGAATGAAC

      1130       1140       1150       1160       1170       1180       1190
         *          *          *          *          *          *          *
AGGGATCAGGCTATGCAGCGGATCAAAAAAGCACACAAAATGCCATTAACGGGATTACAAACAAGGTGAA

      1200       1210       1220       1230       1240       1250       1260
         *          *          *          *          *          *          *
CTCTGTTATCGAGAAAATGAACATTCAATTCACAGCTGTGGGTAAAGAATTCAACAAATTAGAAAAAAGG

      1270       1280       1290       1300       1310       1320       1330
         *          *          *          *          *          *          *
ATGGAAAATTTAAATAAAAAAGTTGATGATGGATTTCTGGACATTTGGACATATAATGCAGAATTGTTAG

      1340       1350       1360       1370       1380       1390       1400
         *          *          *          *          *          *          *
TTCTACTGGAAAATGAAAGGACTCTGGATTTCCATGACTCAAATGTGAAGAATCTGTATGAGAAAGTAAA

      1410       1420       1430       1440       1450       1460       1470
         *          *          *          *          *          *          *
AAGCCAATTAAAGAATAATGCCAAAGAAATCGGAAATGGATGTTTTGAGTTCTACCACAAGTGTGACAAT

      1480       1490       1500       1510       1520       1530       1540
         *          *          *          *          *          *          *
GAATGCATGGAAAGTGTAAGAAATGGGACTTATGATTATCCCAAATATTCAGAAGAGTCAAAGTTGAACA

      1550       1560       1570       1580       1590       1600       1610
         *          *          *          *          *          *          *
GGGAAAAGGTAGATGGAGTGAAATTGGAATCAATGGGGATCTATCAGATTCTGGCGATCTACTCAACTGT

      1620       1630       1640       1650       1660       1670       1680
         *          *          *          *          *          *          *
CGCCAGTTCACTGGTGCTTTTGGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGTTCTAATGGATCTTTG

      1690       1700
         *          *
CAGTGCAGAATATGCATCTGA
```

Location of primers P100 and P200 in the gene of influenza virus A/PR/8 hemagglutinin glycoprotein

Primers P100 and P200 were used to detect the presence of the HA gene. Under PCR conditions outlined in Section 3.2.8, a DNA fragment of about 0.5-kb long was produced after PCR with DNA prepared from FPV-HA.

APPENDIX IV



Schematic representation of nucleic acid vaccines pCMVIA/control and pCMVIA/H1

CMV-int A, promoter-enhancer and intron A sequences of cytomegalovirus; t-PA, peptide signal of human tissue plasminogen activator; BGH polyA, polyadenyl sequences of bovine growth hormone gene; HA, hemagglutinin gene of influenza virus A/PR/8

ADDENDUM

ERRATA

p1, line 5:	**delete** 'in' after '... immune system which'
p2, line 13:	**replace** 'recognises' with 'recognise'
p2, line 19:	**insert** 'Bjorkman *et al.*, 1987a and 1987b;' before 'Zhang *et al.*, 1992)'
p2, line 24:	**replace** 'proteosomes' with proteasomes'
p16, line 11:	**insert** 'CD40' after '... a ligand for'
p16, line 12:	**delete** 'receptor' after 'TNF'
p16, line 15:	**replace** 'tumour' with 'transforming'
p19, line 23:	**replace** 'The nature and quantity of antigen has also influenced the development of cell-mediated or antibody responses' with 'The development of cell-mediated or antibody responses is also influenced by the nature and quantity of antigen'
p26, line 17:	**replace** 'the virus' after '... does not always neutralise' with all viruses, for example paramyxovirus'
p26, line 21:	**insert** 'respiratory syncytial' after '... affords protection against'
p30, line 3:	**replace** 'bacterial' with 'bacteria'
p30, line 15:	**replace** '... such as atypical measles may arise following vaccination with formalin-treated measles virus' with '... for example atypical measles may arise following measles virus infection of individuals vaccinated with formalin-treated measles virus'
p45, line 18:	**replace** 'augment' with 'augmented'
p64, line 1:	**insert sentence** 'One LD_{50} represents the quantity of influenza virus required to kill 50% of infected mice (n=6) within 1 week post-infection.' before 'Intravenous inocula ...'
p65, line 15:	**insert** 'or anti-FPV' after 'Specific anti-HA'
p65, line 16:	**insert** 'or FPV (10^3 PFU/ml)' after '... influenza virus (5 HAU/ml)'
Table 3.3, footnote c:	**delete** 'effector' after '... the restimulated'
	insert 'for the CTL assay' after 'used'
p85, line 10:	**insert** 'alone' after '... pCMVIA/H1 i.m.'
p96, lines 26 and 27:	**replace** 'flourochrome' with 'fluorochrome'
p97, lines 2,6,7,15 & 16:	**replace** 'flourochrome' with 'fluorochrome'
p118, line 12:	**insert** 'for use in cancer therapy' after 'cytokines'
	delete 'and used for cancer prophylaxis'
p122:	**insert** the following references before 'Blanden, 1970'

Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley. 1987a. Structure of the human class I histocompatibility antigen, HLA-A2. Nature **329**:506-512.

Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley. 1987b. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature **329**:512-518.

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Vol. 68, No. 12

Selective Induction of Immune Responses by Cytokines Coexpressed in Recombinant Fowlpox Virus

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Received 28 July 1994/Accepted 7 September 1994

Avipoxviruses have recently been studied as potential vectors for the delivery of heterologous vaccine antigen. Because these viruses abortively infect mammalian cells yet still effectively present encoded foreign genes to the host immune system, they offer a safer but effective alternative to other live virus vectors. We have examined the effect of coexpressing the cytokine interleukin-6 or gamma interferon on immune responses to a recombinant fowlpox virus expressing influenza virus hemagglutinin. The encoded cytokine was expressed for prolonged periods in infected cell culture with little cytopathic effect due to the abortive nature of the infection. In mice, vector-expressed cytokine dramatically altered immune responses induced by the coexpressed hemagglutinin antigen. Expression of interleukin-6 augmented both primary systemic and mucosal antibody responses and primed for enhanced recall responses. In contrast, expression of gamma interferon markedly inhibited antibody responses without affecting the generation of cell-mediated immunity. The safety of these constructs was demonstrated in mice with severe combined immunodeficiency, and no side effects due to cytokine expression were observed. In summary, fowlpox virus vectors encoding cytokines represent a safe and effective vaccine strategy which may be used to selectively manipulate the immune response.

Fowlpox virus (FPV), a member of the *Avipoxvirus* genus with a double-stranded DNA genome of about 300 kb (12), has recently been developed as a live virus vector for delivery of antigens both to its natural avian hosts (17, 26) as well as a potentially safe vaccine vector to mammalian hosts (13). Although FPV replication is blocked in mammalian cells (35, 38), foreign genes under transcriptional control of early promoters are expressed, resulting in presentation of heterologous protein to the immune system (13, 39). Recently, immunization of humans with vaccines based on recombinant canarypox virus, another avipoxvirus, has been reported (8).

Effective vaccination lies in the development and maintenance of long-term protective immunity. For many diseases, protection is determined by the type of immune response induced. Whether the immune response is driven toward humoral or cell-mediated responses is critically influenced by the profile of cytokines produced by subpopulations of T helper cells (25). To modify the immune responses elicited by vaccine antigens, we have constructed recombinant vaccinia viruses (rVVs) engineered to express cytokine genes (27–30). Results from studies with these rVVs suggest that the encoded cytokines either alter the pathogenicity of the vector virus (20, 30) or selectively augment immune responses generated toward the coexpressed heterologous antigen (27–29).

Here, we describe (i) the construction of recombinant FPV (rFPVs) coexpressing the hemagglutinin (HA) gene of influenza virus A/PR/8 together with the gene for murine interleukin-6 (IL-6) or gamma interferon (IFN-γ) and (ii) the influence of these virus-encoded factors on immune responses. Strong systemic anti-HA cytotoxic T lymphocyte (CTL) responses were generated upon restimulation of splenocytes from rFPV-primed mice; however, the levels of these responses were not influenced by the coexpressed cytokines. When given intranasally (i.n.), anti-HA CTL responses were also elicited in the lungs at similar levels by all of the recombinant viruses. Systemic and mucosal anti-HA antibody responses were selectively augmented by the expression of IL-6, as were recall responses following boosting with rFPV or challenge with wild-type influenza virus. Expression of IFN-γ, on the other hand, suppressed antibody responses at the mucosae but did not affect the generation of CTL responses. These findings have implications for the development of improved vaccination strategies.

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MATERIALS AND METHODS

Mice. SCID (severely combined immunodeficient) and CBA/H mice, 6 to 8 weeks old, were raised under specific-pathogen-free conditions in the Animal Breeding Establishment of the John Curtin School of Medical Research.

Cells. Chicken embryo skin cell monolayers were prepared as described previously (7). Human 143B cells (American Type Culture Collection, Rockville, Md.) and murine L929 cells were grown in F15 medium (GIBCO, Grand Island, N.Y.) supplemented with 5% fetal calf serum (Flow Laboratories, North Ryde, Australia).

Viruses. FPV-M3, a tissue culture passage strain derived from the mild vaccine strain FPV-Web (Arthur Webster Pty. Ltd., Northmead, Australia), was grown in chicken embryo skin cell monolayers as described previously (7). Influenza virus A/PR/8 and rVV were grown and purified according to standard techniques (19, 20).

DNA manipulation and sequencing. Molecular cloning, restriction enzyme digestion, and other recombinant DNA techniques were performed by conventional procedures (33). For sequencing, single-stranded DNA templates were prepared from recombinant phagemid pTZ19U and sequenced by the dideoxy-chain termination method of Sanger et al. (34), using the PRISM Ready Reaction DyeDeoxy Terminator Cycle

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sequencing kit (Applied Biosystems, Inc., Foster City, Calif.). Analysis of DNA sequences was performed with SeqEd version 1.0 (Applied Biosystems, Inc.).

PCR primers P330 (5′TAGGTATAGACGAGGC3′) and P348 (5′ATCGAACTCCATTCCG3′) homologous to the FPV *tk* gene and the *Nco*I site downstream of the *tk* gene (17), respectively, were used for PCR analysis of rFPV genome arrangements.

Primers PHA-1 (5′ACTGGATCCATGAAGGCAAACCT A3′) and PHA-2 (5′ACTGGATCCAGATCTAAAAAAAAT CAGATGCATATTCTGCACTGCAAAGATCC3′), complementary to the 5′ and 3′ ends of the open reading frame of the HA gene of influenza virus A/PR/8, respectively, were designed with *Bam*HI and *Bgl*II restriction sites for PCR fragment subcloning. PHA-2 also contains two overlapping transcriptional termination signals, T8 (4), and an in-frame translational stop codon for termination of expression at the 3′ end.

PIL6-1 (5′ACTGGATCCATGAAGTTCCTCTCT3′) and PIL6-2 (5′ACTGGATCCAAAAAAAACTAGGTTTGCCG AGTAGATCTCAAAGTGAC3′), containing transcriptional and translational stop signals and *Bam*HI restriction sites for subcloning of PCR fragments, were used for preparation of murine IL-6 gene for subcloning.

For subcloning the murine IFN-γ gene, primers PIFNG-1 (5′ ACTGGATCCATGAACGCTACACACTGCATCTTGG CT3′) and PIFNG-2 (5′ACTGGATCCAAAAAAAATCAG CAGCGACTCCTTTTCCGCTTCCT3′), with similar features to those of the primers for IL-6, were used.

Isolation of rFPVs. rFPVs were constructed as described elsewhere (6), using the coexpressed *Escherichia coli gpt* gene for recombinant virus amplification and selection (5). Recombinant viruses (containing a *lacZ* gene) were then purified by plaquing under nonselective conditions with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Bethesda Research Laboratories, Gaithersburg, Md.). DNA from recombinant viruses was analyzed by PCR, as described previously (17), to confirm both the predicted structure of the genome and the absence of wild-type FPV-M3.

Cytotoxicity assays. Cytotoxic activity was measured in ^{51}Cr release assays (20). L929 target cells were infected with wild-type FPV-M3, rFPV, or rVV at a multiplicity of infection of 10 or with 5×10^{-3} hemagglutinating units of influenza virus per cell during ^{51}Cr labeling.

Antibody assays. Anti-HA-specific antibodies were assayed by enzyme-linked immunosorbent assay (ELISA) (3). Anti-HA antibody-secreting cells (ASC) among lung and spleen lymphoid cell isolates were enumerated by using an ELISPOT assay described elsewhere (28).

Cytokine assays. Supernatants from cell cultures infected with rFPV were assayed for IL-6 by [^{3}H]thymidine incorporation by using the IL-6-dependent cell line B9 (1) or for IFN-γ by indirect ELISA, as described elsewhere (9), using the purified rat anti-murine IFN-γ antibody R4.6A2 as the capture antibody and a polyclonal rabbit anti-murine IFN-γ antibody as the secondary antibody.

RESULTS

Construction of FPV plasmid insertion vectors. A novel FPV insertion plasmid, pLRB100, was derived from pAF09 (17) (Fig. 1). Plasmid pLRB100 contains the HA gene inserted as a 1.8-kb *Bgl*II fragment proximal to the early promoter of an FPV bidirectional promoter, P.E/L (23), and an additional copy of the P.E/L promoter at the 3′ end of the HA gene. In this plasmid, the ATG initiation codon of HA is preceded by four codons in alignment to the ATG initiation codon of the P.E/L promoter. In addition, pLRB100 contains a *Bam*HI site in front of the second P.E/L promoter for further insertion of cytokine genes.

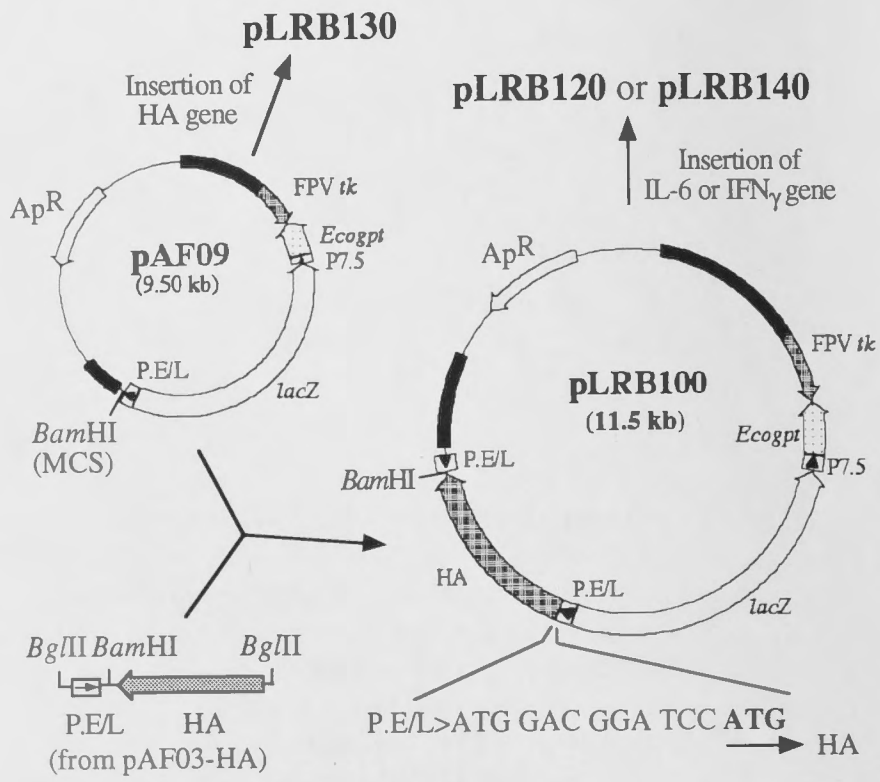

FIG. 1. Schematic illustrations of the construction of FPV insertion plasmids pLRB100, pLRB120, pLRB130, and pLRB140. The HA gene was amplified by PCR from pJZ102 (40), using PCR primers PHA-1 and PHA-2, and subcloned into pAF03. The HA gene and an inverted repeat P.E/L promoter at the 3′ end flanking a *Bam*HI site were recovered as a 1.8-kb *Bgl*II fragment. This fragment was then subcloned into pAF09 (17) to produce pLRB100, a novel FPV insertion plasmid vector into which a *Bam*HI fragment of murine IL-6 or IFN-γ was then subcloned. Plasmid pLRB120 contains a copy of the IL-6 gene, derived from pCD-muIL6 (10) by PCR using primers PIL6-1 and PIL6-2, at the *Bam*HI site of pLRB100. Insertion of an IFN-γ gene, amplified from pCD-muIFNγ (16), at the *Bam*HI site of pLRB100 gave rise to pLRB140. A control virus expressing only HA but no cytokine genes, pLRB130, was constructed by insertion of a PCR-derived HA gene into pAF09. *Ecogpt*, *E. coli gpt*; MCS, multiple cloning site.

Plasmids pLRB120 and pLRB140 contain a copy of PCR-derived IL-6 and IFN-γ genes, respectively, at the *Bam*HI site of pLRB100. Insertion of a PCR-derived HA gene into pAF09 gave rise to pLRB130 (Fig. 1). Nucleotide sequences of the PCR-derived HA, IL-6, and IFN-γ genes were confirmed by sequencing.

Expression of HA and cytokine genes by rFPVs in vitro. Transfection of FPV-M3-infected chicken embryo skin cells with plasmid insertion vectors pLRB130, pLRB120, and pLRB140 resulted in isolation of recombinants FPV-HA, FPV–HA–IL-6, and FPV-HA–IFN-γ, respectively. The genomic arrangement of these rFPVs was confirmed by PCR and Southern blot analyses (data not shown) and is illustrated in Fig. 2. Expression of HA on the surface of rFPV-infected human 143B cells was shown by immunofluorescent staining (data not shown), while secretion of biologically active IL-6 and IFN-γ was confirmed by bioassay and ELISA, respectively (Table 1). Biological activity of secreted IFN-γ was confirmed by bioassay using WEHI-279 cells (31) (data not shown).

Table 1 shows daily production of IL-6 and IFN-γ from 143B cells infected with either rFPV or rVV. Expression of these factors was more protracted in cultures infected with rFPV. Microscopic examination at day 3 after inoculation revealed

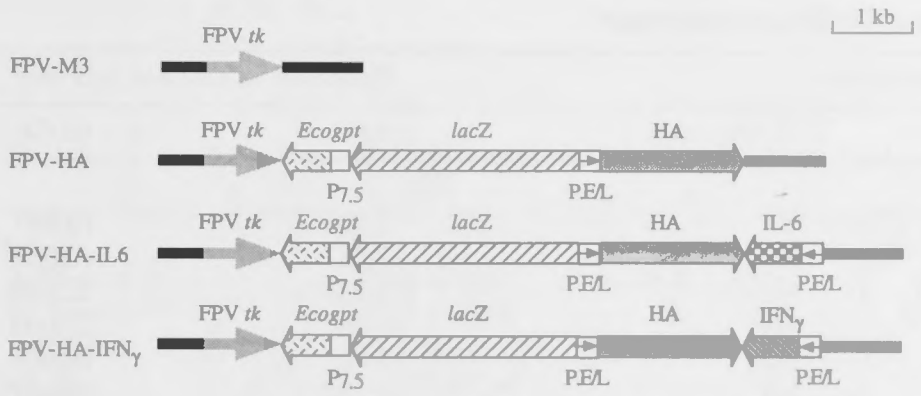

FIG. 2. Genomic configuration of rFPVs. Foreign genes were inserted at a single site downstream of the *tk* gene. *E. coli gpt* (*Ecogpt*; under control of a VV P7.5 promoter) and *lacZ* (under control of the late P.E/L promoter) genes were used for selection and purification, respectively, of rFPV. For the control virus FPV-HA, the HA gene of influenza virus was subcloned proximal to the early/late P.E/L promoter. FPV–HA–IL-6 and FPV–HA–IFN-γ contain the murine IL-6 and IFN-γ genes, respectively, each under the transcriptional control of early/late P.E/L promoters. The HA, IL-6, and IFN-γ genes contain transcriptional stop signals at 3′ ends. The use of inverted repeats of the identical promoter, in this case P.E/L, ensures the stability of recombinants (36). To achieve this, HA and cytokine genes in rFPV were expressed in opposite directions and independently of each other by inclusion of transcriptional terminator signals at 3′ end of each gene.

that the rVV-infected cells had undergone cell lysis, while rFPV-infected cells, which are abortively infected, were still viable.

SCID mice survive infection with rFPVs. SCID mice lack functional B and T lymphocytes and are therefore highly susceptible to virus infection. These mice were used to test the safety of rFPV. Pathogen-free SCID mice were inoculated intravenously (i.v.) with up to 10^8 PFU of FPV-HA or FPV–HA–IL-6 and were monitored for up to 3 months postimmunization. No mortality was observed in any mice given rFPV. Histological examination of tissues from spleen, ovaries, lungs, and liver obtained from infected mice revealed no obvious pathological changes (data not shown).

Induction of systemic CTL responses. No significant primary splenic CTL responses against the FPV vector or coexpressed HA were found at either 1 or 4 weeks after i.v. inoculation with FPV-HA, FPV–HA–IL-6, or FPV–HA–IFN-γ (data not shown). In contrast, strong CTL responses against HA were found among splenocytes taken 3 weeks postimmunization and restimulated with influenza virus in vitro. For clarity, only data obtained from mice given control virus, FPV-HA, are shown

TABLE 1. Daily in vitro production of cytokines[a]

24-h period	U of cytokine/ml of supernatant from cells infected with:			
	FPV–HA–IL-6[b]	VV–HA–IL-6[b]	FPV–HA–IFN-γ[c]	VV–HA–IFN-γ[c]
24	65,301	31,512	650	1,175
48	21,541	9,360	370	126
72	9,057	348	117	<5
144	4,374	<100	68	<5

[a] Supernatants from 143B monolayers infected at a multiplicity of infection of 1 were passed through 0.1-μm-pore-size filters to remove virus particles.
[b] IL-6 was assayed by using the IL-6-dependent cell line B9. IL-6 was detected at very low background levels from 143B cells infected with a control virus, either FPV-HA or VV-HA (2).
[c] IFN-γ activity was assayed by ELISA. No IFN-γ was detected in supernatants overlying cells infected with control viruses.

TABLE 2. Systemic CTL responses in mice given FPV-HA[a]

In vitro restimulation	Specific lysis[b] of L929 cells infected with:			
	No virus	FPV-M3	Influenza virus	VV(WR)
Nil	4.9	5.6	15.6	2.7
FPV-HA	14.1	86.1	82.0	18.6
A/PR/8	4.6	26.0	73.7	16.0

[a] Groups of four mice were given 10^7 PFU of FPV-HA i.v. Spleen cells taken from mice at week 3 postimmunization were restimulated in vitro for 5 days.
[b] Thirty-three percent of the restimulated effector cell cultures were used. The standard error of the mean was <5%.

(Table 2). Moreover, in vitro restimulation of effectors with the corresponding rFPV used for priming also resulted in marked anti-HA CTL responses and responses against the vector. No difference in the level of secondary CTL responses was observed when splenocytes were taken from mice given rFPV expressing IL-6 or IFN-γ. In addition, FPV-primed effectors were unable to lyse VV-infected target cells, even after secondary restimulation, pointing to a lack of cross-reactivity of FPV and VV.

Modest anti-HA and anti-FPV CTL responses were found in mice boosted at week 3 with rFPV. Boosting with VV-HA after priming with FPV-HA also induced anti-HA CTL responses (data not shown). Again, no effect due to the coexpression of IL-6 or IFN-γ was observed.

Induction of systemic antibody responses. Mice were given FPV-HA or FPV–HA–IL-6 i.v., and primary anti-HA antibody responses were monitored for 12 weeks. At 1 week after priming, responses mounted in mice given FPV–HA–IL-6 were twofold higher than those measured in animals given FPV-HA; however, little difference in the levels of antibody responses was noted later (data not shown). Anti-FPV antibody titers were low. Both anti-HA and anti-FPV immunoglobulin G (IgG) titers were enhanced after boosting with rFPV, while recall anti-HA antibody titers were markedly elevated upon challenge with wild-type influenza virus. The higher levels of anti-HA antibody were sustained for at least 9 weeks after immunization with the IL-6-expressing rFPV.

Following subclass analysis of these responses, it was found that the IL-6-expressing rFPV stimulated heightened IgG1 responses, which were maintained for at least 4 weeks (Table 3). However, boosting with rFPV or challenge with wild-type influenza virus induced high levels of IgG2a antibody, which accounted for the great majority of the IgG response.

Induction of mucosal immune responses. While no significant antibody responses were detected in spleens of mice given rFPV i.n. (data not shown), marked differences in mucosal antibody responses were found in mice. Strong IgA and IgG responses were found in the lungs of mice given FPV–HA–IL-6 by 1 week after immunization, whereas low responses were detected in mice given control virus (Table 4). IgA responses were two- to sixfold higher in mice given FPV–HA–IL-6 than in controls, while the increase in IgG was less pronounced. Interestingly, coexpression of IFN-γ suppressed mucosal antibody responses.

Mucosal antibody responses were greatly augmented following boosting with FPV–HA–IL-6 but, again, were suppressed in mice rechallenged with FPV–HA–IFN-γ (Table 5). Mucosal anti-HA antibody responses in mice primed with FPV–HA–IL-6 were also greatly enhanced following challenge with wild-type influenza virus (Table 6).

CTL responses were detected at similar levels in the lungs of mice given each of these rFPV (data not shown).

TABLE 3. Systemic anti-HA IgG subclass responses[a]

Time	Immunization		Reciprocal of endpoint IgG titer	
	Primary	Secondary	IgG1	IgG2a
Primary responses				
Wk 1	FPV-HA	Nil	2,133	10,667
	FPV–HA–IL-6	Nil	17,067	10,067
Wk 4	FPV-HA	Nil	5,333	25,600
	FPV–HA–IL-6	Nil	10,667	25,600
Wk 12	FPV-HA	Nil	1,867	10,667
	FPV–HA–IL-6	Nil	2,133	10,667
Boosting at 3 wk after immunization				
Wk 1	FPV-HA	FPV-HA	34,133	204,800
	FPV–HA–IL-6	FPV–HA–IL-6	59,733	273,067
Wk 9	FPV-HA	FPV-HA	5,333	10,667
	FPV–HA–IL-6	FPV–HA–IL-6	5,333	34,133
Challenge at 3 wk after immunization				
Wk 1	FPV-HA	Influenza virus	34,133[b]	170,667[c]
	FPV–HA–IL-6	Influenza virus	29,867[b]	204,800[c]
Wk 9	FPV-HA	Influenza virus	3,733[d]	25,600[e]
	FPV–HA–IL-6	Influenza virus	10,667[d]	42,667[e]

[a] Groups of four mice were given 10^7 PFU of virus i.v., and antigen-specific antibody responses were determined at time intervals as indicated. At week 3 postimmunization, mice were given a booster, similar to the priming dose, or challenged with 10^3 hemagglutinating units of wild-type influenza virus i.v. Recall antibody responses were determined 1 or 9 weeks later.
[b] Titer in control groups that were not given rFPV prior to challenge was <400.
[c] Titer in control groups that were not given rFPV prior to challenge was 25,600.
[d] Titer in control groups that were not given rFPV prior to challenge was <400.
[e] Titer in control groups that were not given rFPV prior to challenge was 6,667.

DISCUSSION

We have previously shown that the immune response can be markedly influenced by cytokines expressed by rVV (20, 27–30). In the present study, we have investigated whether the expression of cytokines can also influence immune responses to FPV, a nonreplicating viral vector. We found that recombinant FPV vectors expressing HA induced poor primary splenic CTL responses, although strong secondary responses could be generated following restimulation in vitro. This finding indicates that anti-HA CTL precursors were generated following the primary infection. Levels of secondary CTL responses were not influenced by virus-encoded IL-6 or IFN-γ, however, suggesting that optimum levels of these cytokines, if required, are already provided by the host immune system. Although CTL responses were not apparently influenced by virus-encoded cytokines, antibody responses against the heterologous antigen were markedly and selectively augmented by IL-6 and were suppressed by IFN-γ. The IL-6-encoding virus induced a heightened IgG response which was predominantly of the IgG1 subclass. The preferential induction of this antibody subclass is consistent with our previous findings (30) and with other in vivo actions of this cytokine (37). Interestingly, on subsequent boosting or challenge with wild-type influenza virus, the majority of the HA-specific IgG response was of the IgG2a subclass, which is typically elicited by virus infection (18). Although virus-encoded IL-6 did not alter peak levels of HA antibody, the response declined much more slowly in mice given FPV–HA–IL-6 than in those given control virus. Thus, 9 weeks after the last boost, antibody titers against the HA antigen were two- to threefold higher in mice given FPV–HA–IL-6. It is not clear how IL-6 might influence the longevity of the response, given that this molecule is thought to act on terminally differentiated plasma cells (22). A possible explanation is that there is low but prolonged expression of the heterologous genes encoded in rFPV during clearance of the virus. We are currently investigating the stability of FPV-encoded genes in inoculated animals.

TABLE 4. Primary mucosal anti-HA antibody responses[a]

Time (wk)	Immunization	No. of anti-HA ASC/10^6 cells (mean ± SD)	
		IgG	IgA
1	FPV-HA	50.4 ± 17.3	21.0 ± 6.0
	FPV–HA–IL-6	>200	122.0 ± 18.0
	FPV–HA–IFN-γ	<10^b	7.0 ± 2.6
2	FPV-HA	20.0 ± 1.7	13.3 ± 1.2
	FPV–HA–IL-6	20.0 ± 1.0	33.3 ± 2.3
	FPV–HA–IFN-γ	<10^b	<10^b
3	FPV-HA	32.3 ± 2.4	10.2 ± 2.9
	FPV–HA–IL-6	41.2 ± 3.1	21.4 ± 4.2
	FPV–HA–IFN-γ	<10^b	<10^b

[a] Groups of four mice were given 10^7 PFU of virus i.n., and anti-HA ASC in the lungs were enumerated at the times indicated. The data shown are from a single experiment representative of three such experiments.
[b] Limit of detection.

More dramatic enhancement of antibody responses was demonstrated when the recombinant IL-6 FPV was administered i.n. Anti-HA ASC appeared earlier at mucosae and at significantly elevated levels in mice given FPV–HA–IL-6. We have previously shown that antibody responses at mucosae are critically dependent on IL-6, as mice with targeted disruption of the IL-6 gene mount negligible such responses following antigenic challenge (27). These responses are fully restored, however, following challenge with rVV expressing IL-6. In the experiments reported here, the supplementation of host-derived IL-6 by FPV-encoded cytokine clearly enhances the mucosal antibody response. Antibody responses peaked early and were sustained at higher levels in mice boosted with FPV–HA–IL-6 than control virus. FPV-encoded IL-6 also primed for enhanced local antibody responses to coexpressed HA antigen upon subsequent i.n. challenge with wild-type influenza virus, a finding of potential importance for resistance to infection. Together, these results suggest an important role

TABLE 5. Recall mucosal anti-HA antibody responses[a]

Virus	No. of anti-HA ASC/10^6 cells (mean ± SD) at wk:					
	1		2		3	
	IgG	IgA	IgG	IgA	IgG	IgA
Expt 1[b]						
FPV-HA	93.3 ± 14.0	40.0 ± 12.0	85.0 ± 30.0	30.0 ± 17.0		
FPV–HA–IL-6	486.7 ± 11.9	1,320 ± 52.9	180.0 ± 47.5	163.5 ± 55.5		
FPV–HA–IFN-γ	<20[c]	<20[c]	<20[c]	<20[c]		
Expt 2[d]						
FPV-HA	34.3 ± 6.2	33.3 ± 4.5			9.3 ± 2.2	37.3 ± 3.6
FPV–HA–IL-6	262.2 ± 52.1	496.7 ± 70.1			100.3 ± 13.2	142.0 ± 9.1
FPV–HA–IFN-γ	ND[e]	ND			ND	ND

[a] Groups of four mice were given 10^7 PFU of virus i.n. and an i.n. booster similar to the priming dose at the times indicated. Recall antibody responses in the lungs were determined as indicated. In each case, data are from a single experiment representative of three such experiments.
[b] Mice were boosted at 1 week postimmunization, and recall antibody responses in the lungs were determined at 1 and 2 weeks after boosting.
[c] Limit of detection.
[d] Mice were boosted at 3 weeks postimmunization, and recall antibody responses in the lungs were determined at 1 and 3 weeks after boosting.
[e] ND, not determined.

for IL-6 in the expansion of immune cells during the development of the immune response. Therefore, strategies for the delivery of IL-6, such as described in this report, may have major benefits in the development of improved mucosal vaccines.

Our results clearly show that expression of virus-encoded IFN-γ suppressed antibody responses at the mucosae. Greatly reduced systemic antibody responses were also observed in mice given i.v. inocula of IFN-γ-expressing rFPV despite increased cellularity of the spleen (unpublished data). Moreover, systemic anti-VV antibody titers were significantly lower in mice infected with IFN-γ-expressing rVV (21). While mucosal antibody responses were markedly suppressed in the present study, CTL responses to FPV–HA–IFN-γ were as high as those generated by control virus. The ability to selectively generate a strong cell-mediated immune response may have important implications in the development of vaccines against intracellular pathogens. For example, it has been suggested that the preferential induction of such responses may be of paramount importance for successful vaccination against human immunodeficiency virus (32).

An important factor governing the use of vaccine vectors is the question of safety. Because avipoxviruses abortively infect mammalian cells, they offer, in theory, a safer alternative to replication-competent viruses. Although further testing should be undertaken, we have demonstrated the safety of rFPV in a model of immunodeficiency (SCID mice). Moreover, the coexpression of cytokines did not modify this feature of the vector.

Interestingly, the nature of the abortive infection may be an important element in promoting immune responses toward the heterologous antigen. Failure to produce FPV proteins would be expected to reduce antigenic competition between viral and heterologous protein, and the immune response would, therefore, be directed toward the encoded antigens driven by early promoters. The poor immune response mounted against FPV may also retard clearance of the virus, allowing more prolonged expression of the vaccine antigens. Furthermore, booster immunizations are likely to be more effective in situations in which there is a reduced primary immune response to the vector itself. Indeed, prior exposure to the vector did not inhibit boosting of antibodies against rabies virus glycoprotein by recombinant canarypox virus expressing this antigen in humans (8).

The finding that there was no immunological cross-reactivity between FPV and VV may have important practical applications. A major problem in using VV as a vaccine vector is that a proportion of the population have already been immunized with the virus during the smallpox eradication campaign (15). In consequence, these individuals mount lower antibody responses to coexpressed vaccine antigens when given rVV than their nonvaccinated counterparts (11, 14). Our results suggest that this would not be a problem if FPV is used as a vaccine vector. Furthermore, it may be possible to use these different vectors encoding the same vaccine antigen to enhance the efficacy of multiple immunizations. Other vectors have been combined successfully in this respect (24).

In summary, our results demonstrate not only the efficacy and safety of FPV vectors but also the use of coexpressed cytokines to selectively manipulate the immune response.

TABLE 6. Mucosal antibody responses after challenge with influenza virus[a]

Time (wk) postchallenge	Immunization	No. of anti-HA ASC/10^6 cells (mean ± SD)	
		IgG	IgA
1[b]	FPV-HA	59.0 ± 15.6	95.7 ± 47.9
	FPV–HA–IL-6	312.2 ± 23.4	569.7 ± 72.6
3[c]	FPV-HA	11.0 ± 4.1	28.8 ± 9.2
	FPV–HA–IL-6	58.3 ± 17.2	97.3 ± 5.5

[a] Groups of four mice were immunized with 10^7 PFU of virus i.n., and at 3 weeks postimmunization, mice were challenged with a sublethal dose (10^{-3} hemagglutinating units) of wild-type influenza virus i.n. Recall antibody responses in the lungs were determined as indicated. The data shown are from a single experiment representative of three such experiments.
[b] Anti-HA IgG and IgA ASC numbers in control mice that were not primed with rFPVs before challenge were 5.4/10^6 and 9.7/10^6 lung cells, respectively.
[c] Anti-HA IgG and IgA ASC numbers in control mice that were not primed with rFPVs before challenge were 12.3/10^6 and 27.2/10^6 lung cells, respectively.

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